Relationships Between the Sarcoplasmic Reticulum and Sarcolemmal Calcium Transport Revealed by Rapidly Cooling Rabbit Ventricular Muscle

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ABSTRACT   Rabbit right ventricular papillary muscles were cooled from 30 to ~1°C immediately after discontinuing electrical stimulation (0.5 Hz). This produced a contracture that was 30–50% of the preceding twitch magnitude and required 20–30 s to develop. The contractures were identical in cooling solutions with normal (144 mM) or low (2.0 mM) Na. They were therefore not Na-withdrawal contractures. Contracture activation was considerably slower than muscle cooling (~2.5 s to cool below 2°C). Cooling contractures were suppressed by caffeine treatment (10.0 mM). Rapid cooling did not cause sufficient membrane depolarization (16.5 ± 1.2 mV after 30 s of cooling) to produce either a voltage-dependent activation of contracture or a gated entry of Ca from the extracellular space. Contractures induced by treating resting muscles with 5×10^{-5} M strophanthidin at 30°C exhibited pronounced tension noise. The Fourier spectrum of this noise revealed a periodic component (2–3 Hz) that disappeared when the muscle was cooled. Cooling contractures decayed with rest ($t_{1/2} = 71.0 \pm 9.3$ s). This decay accelerated in the presence of 10.0 mM caffeine and was prevented and to some extent reversed when extracellular Na was reduced to 2.0 mM. 20 min of rest resulted in a net decline in intracellular Ca content of 1.29 ± 0.38 mmol/kg dry wt. I infer that cooling contractures are principally activated by Ca from the sarcoplasmic reticulum (SR). The properties of these contractures suggest that they may provide a convenient relative index of the availability of SR Ca for contraction. The rest decay of cooling contractures (and hence the decay in the availability of activating Ca) is consistent with the measured loss in analytic Ca during rest. The results suggest that contraction in heart muscle can be regulated by an interaction between sarcolemmal and SR Ca transport.

INTRODUCTION

Mammalian heart muscle contractions are weakened by prolonged rest (Koch-Weser and Blinks, 1963; Edman and Jóhannsson, 1976; Bass, 1976; Allen et al.,

1976). This is often attributed to the depletion of an intracellular store of activating Ca. Some authors (Allen et al., 1976) have proposed that during rest Ca is transported from the lateral cisternal sacs of the sarcoplasmic reticulum (SR) to the outside of the cell. This could occur by one of several mechanisms. For example, if Ca leaks from the SR during rest, then upon arriving in the cytosol it may be either sequestered or transported by a variety of processes. These include the ATP-dependent SR Ca pump, the ATP-dependent sarcolemmal (SL) Ca pump, and SL Na/Ca exchange. If transsarcolemmal Ca efflux is initially greater than influx, rest will induce a net loss of cellular Ca. Mullins (1981) explains how the relative abundance, turnover rate, and Michaelis constants of cell Ca pumps will determine how much of the Ca leaking from the SR is removed from the cell. SL Ca transport may therefore be an important determinant of SR Ca content.

If the Ca content of the SR does determine the strength of contraction (Allen et al., 1976), then transsarcolemmal Ca transport may indirectly regulate contractile strength under a variety of circumstances. During a series of identical twitches, the intracellular Ca content remains unchanged at the end of each cycle of contraction and relaxation. Any Ca entry during this period must therefore be balanced by an equal loss. Transient imbalances in the gain and loss of Ca that occur over a few cycles will lead to transient changes in the intracellular Ca content. The available data indicate that the SR can rapidly accumulate and release Ca (e.g., Levitsky et al., 1981; Fabiato, 1981). It is therefore possible that changes in the intracellular Ca content result in changes in the time-averaged SR Ca content during activity. Anything that reduces Ca extrusion while leaving entry unchanged might actually increase SR Ca content and contractility. Small imbalances in fluxes over one contraction-relaxation cycle might not produce observable effects on tension. However, if the change in intracellular Ca content produced by these imbalances is accumulated by the SR over several cycles, and if it is assumed that changes in SR Ca content alter the amount of Ca available for contraction, these effects may become apparent. Modest changes in transsarcolemmal Ca transport might therefore have profound effects on tension development after a number of beats.

In view of the foregoing ideas, an examination of the relationship between the availability of SR Ca and SL transport is worthwhile. It should be possible to prevent rest-induced loss of SR Ca (and contractility) by preventing Ca transport from the cell. Similarly, increasing cellular Ca entry during rest might replenish the SR Ca stores and reverse any rest-induced loss of SR Ca. These investigations require some physiological index of the availability of SR Ca for contraction, as well as some means of manipulating transsarcolemmal Ca transport.

I first attempted to show that contractures elicited by rapidly cooling heart muscle are predominantly activated by SR Ca. The properties of cooling contractures suggest that their magnitude provides a relative measure of the availability of SR Ca for contraction. If it is assumed that the availability of SR Ca for contraction depends directly on SR content, then the cooling contracture magnitude reflects the SR Ca content. Procedures that interfere with transsarcolemmal Ca transport effectively change the subsequent cooling contracture magni-

tude (and presumably SR Ca content). Finally, I measured the rest-induced loss in analytical Ca using matched pairs of muscles from the same animal. The result from this measurement can account for the observed rest decay of cooling contractures. It also provides an estimate of the minimum quantity of Ca entering a heart cell during a single contraction. An abstract of this work has already appeared (Bridge, 1985).

METHODS

Dissection and Preparation

White New Zealand rabbits, typically weighing <5 lbs, were heparinized and killed with a lethal injection of sodium pentobarbital. The heart was excised and immediately perfused via the aorta in a retrograde fashion with a bicarbonate-buffered, modified Tyrode's solution containing Ca at a concentration of 50 μM. This greatly facilitated dissection. The cannula, which was inserted into the aorta, projected from the side of a Plexiglas dissecting dish. The heart was pinned down to a Sylgard surface at the bottom of the dish and the right ventricular papillary muscles were exposed. Papillary muscles were dissected out while the heart was perfused. Small 8.0 silk loops (the silk was a gift from Davis and Geck, Inc., Danbury, CT) were attached to the tendinous end of the muscles, which were then cut away from the interventricular septum. With rare exceptions, muscles of <1.0 mm diam were used. The muscles were mounted in a thin-walled, rapid-flow Plexiglas muscle chamber similar in design to that described by Gadsby and Cranefield (1977). The flow rates of the superfusing solutions were usually maintained at 5 ml/min. However, during rapid temperature jumps, when higher flow rates were required, the flow rate was ~15.0 ml/min.

The tendinous end of the muscle was attached to the arm of a tension transducer (FT03, Grass Instrument Co., Quincy, MA). The cut end of the muscle was secured by a small nylon (6.0) loop to a theta tubing post cemented into the base of the chamber. In experiments where a pair of muscles from the same heart was used, each one of the pair was mounted in adjacent and identical chambers. The muscles were superfused with a modified HEPES-buffered solution maintained at 30 ± 1 °C. Muscles were stimulated by supramaximal square-wave pulses (duration, 5 ms) at a frequency of 0.5 Hz, applied by platinum field electrodes cemented to the sides of the chambers. Muscles were stretched until maximum twitch tension was observed and equilibrated until stable twitches were obtained. All experiments were carried out at 30°C except when muscles were abruptly cooled to within a couple of degrees of 0°C. At the end of an experiment, the muscle diameter was measured by means of a calibrated reticule in the eyepiece of a stereoscopic microscope (SMZ-2, Nikon, Tokyo, Japan).

Solutions

The following solutions were used in this study. Muscles were dissected in a bicarbonate-buffered Tyrode's solution of the following composition (mM): 126 NaCl, 18 NaHCO$_3$, 5.4 KCl, 0.5 MgCl$_2$, 0.05 CaCl$_2$, 5.5 dextrose, equilibrated with 95% O$_2$, 5% CO$_2$, pH 7.4. Muscles were equilibrated in a HEPES-buffered solution of the following composition (mM): 144.0 NaCl, 1.0 KCl, 4.4 KOH, 0.5 MgCl$_2$, 2.7 CaCl$_2$, 5.5 dextrose, 8.25 HEPES, pH 7.5. The solution was bubbled with 100% O$_2$. In low-Na experiments, Na was reduced to 2.0 mM and replaced with 142.0 mM choline chloride. In experiments involving extracellular space measurement, 1.0 mM KCoEDTA was added to the Tyrode's solution and KCl was reduced by 1.0 mM.

Rapid Temperature Change

The superfusion system designed to rapidly cool small papillary muscles was equipped with two separate thin-walled, rapid-flow muscle chambers. The two chambers (which were required for paired-muscle studies) were positioned adjacent to one another. Muscles were normally superfused with a Tyrode's solution maintained at 30°C. Rapid cooling was achieved by flushing the muscle chamber with a cold Tyrode's solution. An electronically controlled solenoid valve was used to switch to the cooling solution. The main technical difficulty in developing the temperature jump method was to ensure that the temperature of the cold Tyrode's solution did not rise too much before entering the muscle chamber. The superfusing solutions were held in reservoir bottles. Each bottle was connected by gas-impermeable tubing to one of two small drip chambers mounted side by side. The flow rate in this gravity-feed system was monitored using an infrared LED and phototransistor. Adjustments of flow rate were made with a manually operated needle valve in each chamber. The drip chambers break the flow of fluid to the muscle chambers and thus electrically isolate the perfusate reservoirs from the rapid-flow muscle chambers. The reservoir bottles were held in two large (5 gal), double-walled Plexiglas baths. One bath contained 20% propylene glycol and was refrigerated to −4°C by a circulator cooler (A82, Haake Buchler Instruments, Inc., Saddle Brook, NJ). The other bath contained distilled water maintained at 31°C by a circulator heater (A81, Haake Buchler Instruments, Inc.). The gas-impermeable solution delivery lines were individually mounted inside large-diameter Tygon tubing (Norton Plastics and Synthetics Division, Akron, OH), which ran from the Plexiglas baths to a manifold valve mounted on each drip chamber. This fluid from the Plexiglas water baths was pumped through the jacket tubing, then through the walls of the drip chamber (via the manifold), and finally back to the bath. This arrangement ensured that the delivery lines and drip valves were thermally jacketed. Each drip chamber was connected to a rapid-flow bath via a bifurcated piece of Tygon tubing. Despite the thermal jacketing, the superfusing fluid did pick up some heat between the reservoirs and the entrance to the muscle chamber. However, the superfusing solution was always within a degree or two of 0°C. Fluid through the cold dripper was therefore always maintained close to 0°C. A similar jacketing arrangement maintained the temperature of fluid (30°C) through the warm dripper. The manifold valve on top of the dripper permitted the selection of any one of several reservoir bottles in each Plexiglas bath. The rapid-flow baths were machined with thin walls to reduce their thermal capacity, which increased the rapidity with which the temperature jumps were accomplished. Superfusing solutions were continually aspirated from the muscle chamber into a waste bucket. The muscle chamber was illuminated with a fiber optic illuminator (Dolan-Jenner Industries, Inc., Woburn, MA) and observed through a Nikon stereomicroscope. The thermistor used to measure temperature changes had a time constant of <30 ms in flowing solution (15 ml/min).

Membrane Potentials

Membrane potentials were measured with micropipettes filled with 3 M KCl. These pipettes were pulled from borosilicate glass and had resistances between 5 and 20 MΩ. The micropipette was connected via an Ag/AgCl junction of one of the dual inputs of a differential amplifier (input impedance, 10^{15} Ω). A flowing 3 M KCl reference electrode was connected via an Ag/AgCl pellet to the other input of each differential amplifier. The flowing junction was made with a cylindrical ceramic plug (1 mm diam) attached to a KCl reservoir and positioned 5.0 mm downstream from the tissue.

Noise Analysis

Single papillary muscles were attached to the beam of a piezoresistive tension transducer (AME, Horton, Norway) to record tension. This transducer was both AC and DC coupled. The AC- and DC-coupled signals were displayed on separate channels of a chart recorder. The AC-coupled response was observed at high gain and filtered (low pass, 2 Hz; high pass, 10 Hz). The data, which were recorded digitally, were collected for 20 s at a sampling rate of 25 Hz. The digitized data were subject to spectral analysis by fast Fourier transform.

Preparation of Muscles for Elemental Analysis

Pairs of muscles from the same animal were analyzed for Ca content. At the end of an experiment, a pair of muscles was removed from the bath and placed in a Teflon dish
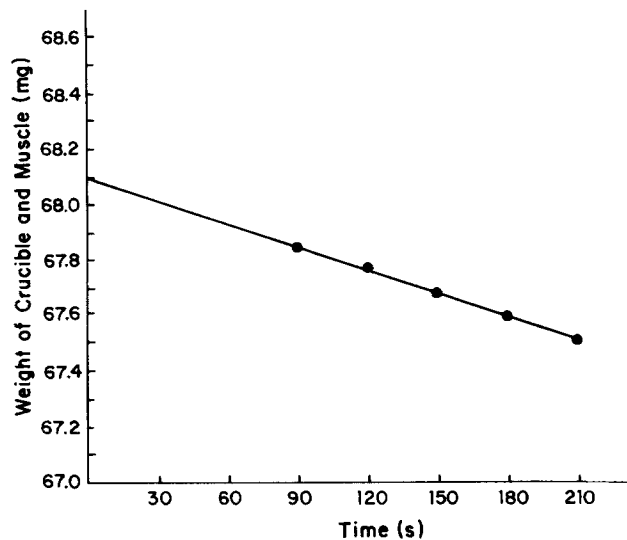
FIGURE 1. Decay of muscle weight (caused by evaporation) during the weighing procedure. The points are extrapolated back to the origin to obtain the muscle weight when the muscle was removed from the bath (see text).

that had been washed with Milli-Q water (Continental Water Systems, El Paso, TX). When a muscle was removed from the bath, a stop watch was started. The tendon and base of the muscle were trimmed and the muscle was conveyed to the weighing pan of a microbalance (Cahn Instruments Inc., Cerritos, CA) using washed forceps. This entire operation did not take more than 90 s. The weight was recorded at 90 s and then every 30 s until at least 240 s had elapsed. The plot of wet weight vs. time was linear. The points were extrapolated to zero time to provide muscle weight at the time the muscle was removed from the bath (Fig. 1). This entire procedure was necessary because small muscles lost moisture rapidly upon exposure to the air. Every time a muscle was weighed on a microcrucible, a control microcrucible that did not contain a muscle was also weighed. Both control and muscle crucibles were placed in washed Teflon vials and dried to constant weight at 100°C. The muscle and control crucible were then reweighed. From the weight of the crucibles, the muscle dry weight, and the extrapolated wet weight, the tissue water, tissue wet weight, and dry weight as a percentage of wet weight were

calculated. The control and muscle-containing crucibles were then placed side by side in a porcelain crucible (also washed) and then placed in a cold muffle furnace. The temperature of the furnace was raised slowly to 450°C and the muscles were allowed to decompose to ash overnight. Both Co and Ca are sufficiently refractory that this temperature produced no loss of these elements during the ashing procedure (Gorsuch, 1970).

Elemental Analysis

PFA Teflon vials were washed and a 0.5% HNO_3 acid solution was added to them. In these experiments, the quantities of Ca to be measured were small and were typically in the range of 200–400 ng/muscle. Extraneous Ca contamination therefore posed a serious threat to the reliability of these measurements. The following precautions were essential to minimize Ca contamination. The use of glassware was avoided because it is a source of Ca contamination. All Teflon and plastic ware (including pipette tips) was refluxed in acid (1:1 HCl) and subsequently in deionized water for several days. This procedure is necessary to wash contaminating Ca from the Teflon. If Ca is not adequately washed from the Teflon, it slowly diffuses out during determinations and contaminates the blanks. This procedure is apparently effective in removing Ca. An extract medium containing 0.5% HNO_3 was made from custom double-distilled HNO_3 (GFS Chemicals, Columbus, OH) and polished Milli-Q reagent water. The extract was stored in uncontaminated Teflon bottles. Only PFA and FEP Teflon were used because they are not porous. The extract was made up in washed plastic volumetrics. The acid was added to the extract with a constant-volume Eppendorf pipette (Brinkmann Instruments Co., Waterbury, NY). One plastic tip was reserved for use with the acid and was uncontaminated. 20-ml PFA Teflon vials (acid washed) were carefully rinsed with polished Milli-Q water. These vials were weighed on a top-loading balance (Mettler Instrument Corp., Hightstown, NJ). 3–5 g (ml) of extract solution was then poured from the Teflon bottle into the vials. If a Ca determination was to be undertaken, these extracts were checked for Ca contamination by atomic absorption spectroscopy using a graphite furnace (to be described in detail later). If the Ca content of the extract exceeded a few percent of the anticipated experimental test reading, the extract was rejected.

The control and ash-containing microcrucibles were placed in separate extract solutions. The ash was allowed to dissolve before a determination was made. Samples were analyzed by atomic absorption spectroscopy. Samples were injected into a GTA95 graphite tube atomizer by means of an auto sampler (both from Varian Associates, Inc., Palo Alto, CA). The auto sampler as supplied by the manufacturer was equipped with glass and plastic sample vials that contributed unacceptably high levels of contaminating Ca, which proved impossible to remove. For this reason, new sample and standard vials were machined out of FEP Teflon. These sample vials, after careful washing, contributed no background contamination and were always tested before a Ca determination. This step made possible measurements of the type and resolution required in this study.

Aliquots of control and ash-containing extract were poured into prechecked auto sample vials and analyzed using a Varian 1470 single-beam atomic absorption spectrophotometer in conjunction with the microprocessor-controlled graphite tube atomizer. Ca determinations were carried out using uncoated graphite atomizer (furnace) tubes, whereas Co measurements were carried out with the more sensitive pyrolytically coated tubes. All furnace parameters were determined empirically.

Measurement of Ca. Determinations were usually carried out on 20-μl samples. Ca absorbance was read at a wavelength of 422 μm. During atomization, the temperature of the furnace was raised to 2,600°C. Fig. 2 displays a typical absorbance change accompanying the atomization of the Ca sample together with the control blank (platinum microcrucible without ashed muscle). It is clear from this example (which is typical) that

the contamination caused by sample handling was insignificant. During Ca determinations, the peak height absorbance rather than the peak area absorbance was used because uncoated graphite atomizer tubes produce a rather broad absorbance profile. Before any determination, standard curves were prepared. The standard solutions were made up in plastic volumetrics using plastic Eppendorf pipettes. A program was written to instruct the autosampler to select suitable aliquots of standard from a single standard solution
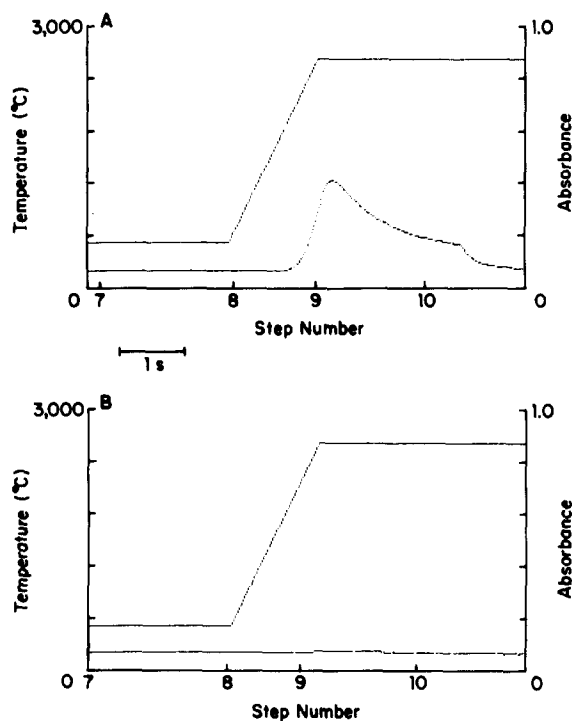

FIGURE 2. Absorbance profile for Ca atomized at 2,600°C in a graphite furnace. The analyte was subjected to a series of programmed temperature steps that culminated in its atomization. The steps just before and during atomization are indicated on the abscissa. A shows the absorbance change produced by Ca in a 20-μl extract of ashed muscle. In B, an extract was made using a dummy crucible that contained no ashed muscle but was otherwise treated identically to the ashed sample. The absence of absorbance indicates that the procedure did not introduce significant levels of contaminating Ca.

(CaCl$_2$ in 0.5% HNO$_3$). The graphite furnace uses a microprocessor to construct a standard curve from the absorbance data. The machine was usually programmed to plot absorbance vs. standard concentration (in nanograms per milliliter). Sample readings were interpolated on this curve. Ca is a refractory element, and the graphite tube exhibits a certain amount of "memory," which can be carried over to the next determination. This was eliminated by incorporating a tube-cleaning program after each atomization step. Phosphate can interfere with the measurement of Ca by atomic absorption spectroscopy. However, at the high atomization temperature used in this analysis, no chemical interference by phosphate was observed.

Measurement of Co. The procedures for measuring Co were similar to those for measuring Ca. The peak area absorbance, rather than the peak height absorbance, produced a more linear standard curve. The extracellular marker KCoEDTA was probably degraded to simple salts during the ashing procedure. However, it is possible that the degradation was incomplete. Standard curves were constructed from CoCl₂ solution (0.5% HNO₃) and KCoEDTA. It is possible that the behavior of CoCl₂ and CoEDTA differed in the graphite furnace. For this reason, the standard curves constructed from KCoEDTA and CoCl₂ were compared. It is clear from Fig. 3 that there is little difference in the two curves, so that the differing molecular forms of Co make little difference in the analysis of Co in the event that KCoEDTA incompletely degrades to simple salts in the graphite furnace.
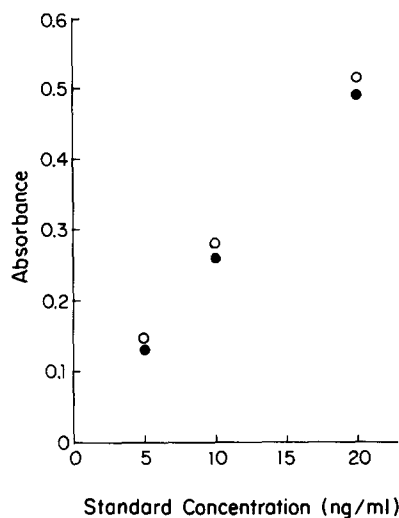

Standard Concentration (ng/ml)

FIGURE 3.   Standard curves established for Co using a graphite furnace. (○) Standard solution made with $CoCl_2 \cdot 6H_2O$. (●) Standard solution made with KCoEDTA · $2H_2O$. The standards contained identical quantities of the element.

Calculations

Results are usually expressed in nanograms per milliliter. From the weight (volume) of the extract, the quantities of Co and Ca residing in the muscle were calculated and converted to molecular quantities. The extracellular KCoEDTA concentration was 1.0 mM. From the quantity found in the muscle, and assuming complete equilibration of the marker in the extracellular space, the quantity of H_2O in the space can be calculated. From this value and the values of wet and dry weight, the apparent intracellular H_2O can be calculated. Intracellular Ca was calculated from the expression:

apparent intracellular Ca [mol] = total tissue Ca [mol] − CoEDTA space [ml]

X extracellular Ca concentration [mol/ml].

This parameter was divided by wet weight, dry weight, or intracellular H_2O. In this study, results are expressed as means ± standard error, unless otherwise stated.

RESULTS

Effect of Abruptly Cooling Rabbit Papillary Muscles

Fig. 4 shows the effect of abruptly cooling a single rabbit papillary muscle. The muscle was first stimulated to contract at a frequency of 0.5 Hz (30°C) in the muscle chamber. After stable twitches were obtained, stimulation was discontinued and the muscle was flushed with a cold, modified Tyrode's solution that was deficient in Na (2.0 mM Na, 142.0 mM choline). This solution reduced the muscle temperature to 1°C (see the next section). The switch to the cooling solution was performed manually and was made to coincide as closely as possible with the last stimulus. This cold flush caused the papillary muscle to develop tension. Muscles typically required between 20 and 30 s to develop maximum
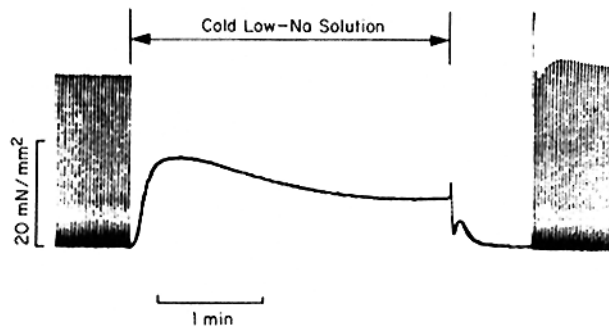

FIGURE 4.   Single rabbit papillary muscle stimulated at 0.5 Hz (30°C). Stimulation was discontinued and the muscle was abruptly cooled to 1°C in a modified Tyrode's solution containing 2.0 mM Na, 142.0 mM choline chloride. This produced a contracture that later declined. After the muscle was exposed to the cooling solution, it was rewarmed to 30°C. After a brief period at 30°C, the muscle was restimulated at a frequency of 0.5 Hz.

contracture tension. The peak magnitude of the contractures usually lay between 30 and 50% of the preceding twitch tension if the contractures were elicited immediately after electrical stimulation.

In most experiments, cooling solutions were deficient in Na (2.0 mM), which was replaced with choline chloride (142.0 mM). I subsequently realized that what I had assumed to be cooling contractures might have been Na-withdrawal contractures. This was checked in the following way. In each of five muscles, cooling contractures were elicited immediately after electrical stimulation. First, a cooling contracture was elicited in a cooling solution deficient in Na (2.0 mM). The muscle was reequilibrated and then cooled in a normal Tyrode's solution (144.0 mM Na). To account for possible deterioration of muscle performance, the procedure was repeated in reversed order. In a total of 10 pairs of observations, the magnitude of cooling contractures elicited in low Na was 0.87 ± 0.12 g (mean $\pm$ SE), and in normal Na, it was 0.86 ± 0.40 g. The mean difference in peak contracture tension in the two pairs of observations was 0.01 ± 0.03 g.

This difference in tension was not significant ($P \leq 0.05$). Therefore, the magnitude of the cooling contractures was independent of the concentration of Na in the cooling solution. Cooling contractures are thus not Na-withdrawal contractures.

Contractures decay incompletely over several minutes. An abrupt rewarming of the muscle produced an abrupt relaxation, accompanied by some tension oscillation. If the muscle is allowed to rest for a short period after rewarming, electrical stimulation produces strengthened twitches that settle down to the precooling magnitude. The effect of cooling on twitch tension is reversible. Muscles can be cooled and rewarmed without injury for many hours during the course of a single experiment. Fig. 5 shows the effect of abruptly heating a
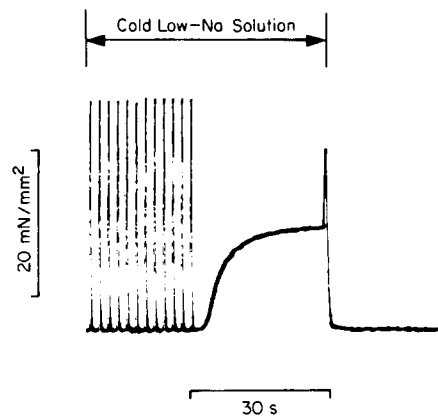

FIGURE 5. Single rabbit papillary muscle stimulated to contract at 0.5 Hz (30°C). Stimulation was discontinued and the muscle was abruptly cooled with a modified Tyrode's solution containing 2.0 mM Na, 142.0 mM choline chloride. At the peak of the cooling contracture, the muscle was reheated, which produced additional contracture tension, followed by prompt relaxation.

muscle at the peak of a cold contracture. There was a rapid increase of tension, immediately followed by relaxation. The peak tension developed averaged 74.8 ± 3.3% (nine observations on three muscles) of the preceding twitch tension. The increase in tension that occurs when muscles are activated in the cold has been described in other preparations (Reiser and Lindley, 1983). There appear to be two possible explanations for the cold-induced contracture of heart muscle. Cooling either elevates free Ca in the vicinity of the myofilaments or increases the sensitivity of the myofilaments to ionized Ca (Godt and Lindley, 1982; Stephenson and Williams, 1981). It is possible that both effects occur together to produce cooling contractures. However, the rapid increase in tension that occurs when a muscle activated in the cold is suddenly heated suggests that cooling reduces the sensitivity of the myofilaments to ionized Ca. Cooling contractures are therefore probably activated by an elevation of cytosolic ionized Ca in the vicinity of the myofilaments.

Time Course of Muscle Cooling

Fig. 6 shows (on an expanded time scale) the relationship between the temperature change of the muscle chamber and the time course of contracture tension. The tip of the thermistor (τ = ~25 ms) used to record temperature decline was placed as close as possible to the surface of the muscle. To achieve rapid temperature changes, the flow rate of the cold solution was increased to ~15.0 ml/min before the switch. The switch to cold flowing solution, which was accomplished manually, was intended to coincide as closely as possible with the last electrical stimulation (the switch is indicated by the arrows in the figure and
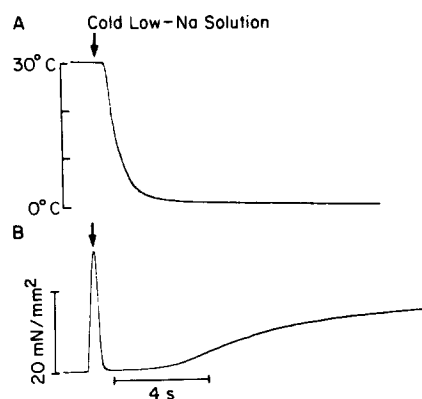

FIGURE 6. (*A*) Time course (expanded scale) with which the muscle bath cooled after a switch (arrow) to a cold (low Na) flowing solution. The temperature change was recorded with a thermistor placed close to the muscle. (*B*) Tension change (expanded scale) after the switch to the cold (low Na) solution. The arrow in *B* indicates when, in relation to the twitch, the switch occurred, which in this case coincided with the peak of the last twitch. This figure shows that bath cooling preceded the development of substantial contracture.

in this case occurred at the peak of the final twitch). There was a brief lag as the chamber dead space cleared. The temperature of the bath then declined rapidly. The onset of contracture was delayed and first became apparent ~2.5 s after the switch. At this time, the bath temperature was 2°C. Tension development continued long after the bath had cooled.

In marked contrast to a twitch at 30°C, the onset and rate of rise of contracture tension were slow when a muscle was immersed in cold solution. It is reasonable to expect the activation of contraction to be markedly slowed at low temperatures. However, the muscle interior must cool more slowly than its surface. This might delay the onset of tension activation and cause it to occur nonuniformly throughout the muscle. Since I knew the rate at which the muscle surface cooled, I was able to calculate the rate at which the interior of the muscle cooled. The heat equation used for this calculation is given in cylindrical coordinates as:

$$\frac{\partial \theta}{\partial t} = \frac{k}{r} \frac{\partial}{\partial r}\left(r \frac{\partial \theta}{\partial r}\right),$$

where θ is the temperature in Kelvins, r is the muscle radius, and $k = K/cp$, where K is the thermal conductivity of muscle, c is the muscle specific heat, and p is muscle density. The values of K, c, and p are given by Hill (1937) as 12.4×10^{-4} cal/cm·s·°C, 1.05, and 0.872, respectively. The rate at which the muscle cooled was calculated by numerical integration (Crank and Nicolson, 1947) of the above equation. The initial condition was

$$\theta = \theta_0 \qquad 0 < r < a \qquad t = 0,$$

and the boundary condition was

$$\theta = \phi(t) \qquad r = a \qquad t \geqq 0.$$

Here θ_0 is the initial bath temperature, a is the radius at the muscle (cylinder) boundary, and $\phi(t)$ is the surface temperature as a function of time.
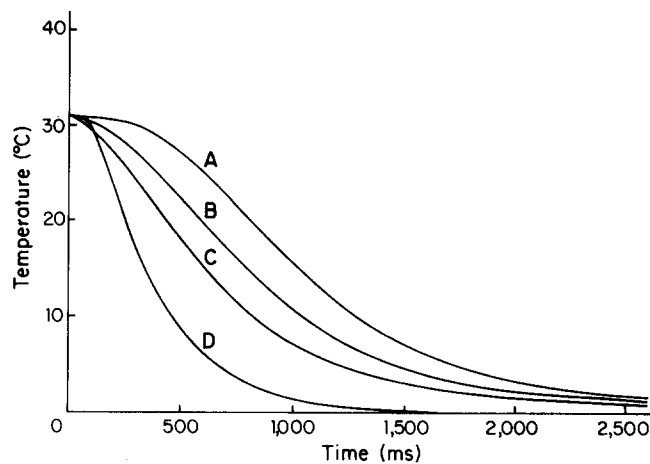

FIGURE 7. Observed and calculated cooling trajectories. Curve C shows the time course with which the surface of a muscle cooled after a switch to low-Na cooling solution. The temperature was recorded with a thermistor placed close to the muscle. Curve A shows the time course with which the core (radius = 0) of the muscle cooled. This was calculated from trajectory C and suitable thermal constants (see Results). Curve B is the calculated decline in average muscle temperature. Curve D is a theoretical time course with which the muscle core would be expected to cool after a step change in bath temperature to 0°C.

The results from this calculation are displayed in Fig. 7. Several muscles yielded almost identical data. Curve C is the function $\phi(t)$, the time course with which the muscle surface temperature declined. In this case, 1.9 s was required to reduce the surface temperature below 2°C. Curve A is the calculated rate at which the muscle core temperature declined. The result shows that 2.4 s was required to reduce the core to below 2°C (the muscle used for this result had a diameter of 0.95 mm). The core temperature therefore lagged the surface temperature by 500 ms. Curve B is the average muscle temperature, which declined below 2°C in 2.3 s and therefore lagged the surface by 400 ms. To

change the muscle temperature to below $5°C$ (when Ca transport processes are likely to be significantly slowed) requires considerably less time than that required to reduce muscle temperature to below $2°C$.

It is possible to calculate the speed of these changes relative to the maximum rate of temperature decline at the core of a muscle of this diameter (0.95 mm). Curve D is the calculated decline in the temperature of the muscle core after a theoretical step change in bath temperature. The core temperature required 930 ms to cool to below $2°C$.

These calculations indicate that the interior of a muscle cooled to below $5°C$ before tension development was apparent. Moreover, tension development continued long after the interior had cooled. The activation of tension appears to be considerably slower than muscle cooling. Any explanation of the activation of tension by cooling must therefore take into account these rather slow activation kinetics and their relationship to temperature changes. If the elevation of cytosolic Ca in the vicinity of the myofilaments explains the development of cooling contractures, then there are two possibilities. The first is that cooling produces an increase in cytosolic Ca that significantly precedes tension development, which is slowed by cooling. The second possibility is that cytosolic Ca increases slowly, with tension development following closely. There is considerable evidence that isolated SR is passively permeable to Ca (e.g., Duggan and Martinosi, 1970; Feher and Briggs, 1982; de Boland et al., 1975). Assuming this to be true of in vivo SR, it follows that the Ca leak from the SR will be uncompensated in the cold (when Ca transporters are immobilized). Ca that leaks from the SR will not be returned to the SR or transported from the cell. Instead, it will slowly rise in the cytosol as it leaks from the SR. The following calculation makes this explanation plausible. Page (1978) has provided data (for rat ventricle) from which the calculated sum of the area of both cisternal and noncisternal SR per unit volume is 1.23 $\mu m^2/\mu m^3$. Inesi (1979) has calculated (from his unpublished data) that the permeability coefficient of the SR is 1.5×10^{-8} cm/s at $37°C$. Assuming that, in the cold, Ca leaks from both cisternal and noncisternal SR, that cytosolic free Ca is 10^{-7} M, that Ca is at a concentration of 10.0 mM inside the SR (Inesi, 1979), and finally that there is no membrane potential across the SR, one can calculate passive flux from the SR as

$$\phi = P_{Ca}(Ca_i - Ca_o),$$

where ϕ is passive flux, P_{Ca} is the SR permeability coefficient, and Ca_i and Ca_o are the intra- and extravesicular Ca concentrations, respectively. Page's estimate of SR surface-to-cell volume was used to calculate that after 20 s of passive Ca flux from the SR, the cytosol would accumulate 36.9×10^{-6} mol Ca/liter of cell water. If 36% of the cell volume is occupied by mitochondria (Page, 1978; Fabiato, 1983), then the concentration of Ca in the Ca-accessible volume would increase to ~50 μM. According to recent calculations by Fabiato (1983), this would activate tension. It is unlikely that passive flux of Ca from the SR will be sustained at the calculated rate for 20 s. As Ca leaves the SR, the leak declines. Moreover, the value for the passive SR permeability used here is for isolated skeletal muscle at $37°C$ and is probably smaller at $0°C$. This will lead to an

overestimation of the effect of Ca leak from the SR in the cold. Despite these uncertainties, this calculation supports the second possibility that the slow activation of cooling contractures is related to a slow leak of Ca from the SR.

Characteristics of Cooling Contractures

The peak magnitude of cooling contracture depends on the behavior of the muscle before cooling. If, for instance, contractures are elicited immediately after electrical stimulation, they are large. If electrical stimulation is discontinued and the muscle is allowed to rest before abrupt cooling, then the ensuing contractures are smaller. The longer the period of rest, the smaller the contractures were, until after a rest of 7 min, the contractures were ~12% of the initial contracture. The rest-induced decay in cooling contracture was measured by first obtaining a maximal contracture immediately after electrical stimulation. After rewarming and reequilibration of the muscle, stimulation was discontinued for 10 s and a contracture was again elicited. Repeating this procedure for increasing rest intervals revealed the time course of rest decay of contractures. This decay is illustrated in Fig. 8. The decay displayed in Fig. 8 was plotted on linear graph paper in Fig. 9. The contracture magnitude at each time was plotted as a fraction of the initial maximum contracture immediately after electrical stimulation. This experiment repeated on five muscles indicated that the $t_{1/2}$ for rest decay of contracture tension was 71.0 ± 9.3 s ($n = 5$). Beyond 7 min of rest, the decay of cooling contracture is very slow.

It has been assumed that cooling contractures are activated by elevations in cytosolic Ca. It follows from this assumption that rest reduces the availability or quantity of Ca that activates these contractures. During rest, the concentration of extracellular Ca remains unchanged. It is therefore reasonable to assume that the availability of extracellular Ca for contracture activation also remains unchanged. Thus, the component of contracture tension that changes with rest is elicited by some intracellular source of Ca. Moreover, the availability of this Ca declines with rest. However, some tension activation by external Ca might occur if cooling effectively immobilizes transsarcolemmal Ca transport, thus allowing a net Ca leak into the cell (see Discussion).

Noise Analysis of Tension During Contracture

The foregoing data suggest that cooling contractures are activated by Ca from some intracellular source and that the availability of this activating Ca declines with rest. This inference hinges upon the assumption that the greater the magnitude of free Ca, the greater the magnitude of the cooling contracture. This assumption may be unwarranted (Orchard et al., 1983). Recent studies indicate that during tonic tension development in heart muscle, both force and cytosolic free Ca oscillate. Orchard et al. (1983) have pointed out that if oscillations are not synchronized between different cells, the muscle tension will be smaller than the mean isometric tension of each cell. Under these circumstances, the relationship between tension development and free Ca concentration is not straightforward.

Single papillary muscles were equilibrated in a standard Tyrode's solution at 30°C. Tension was recorded with a piezoresistive transducer. The tension

transducer was AC coupled to observe tension noise and DC coupled to observe contracture tension. The AC signal was observed at high gain and the DC signal at lower gain (Fig. 10). The noise produced with a resting muscle attached to the transducer was observed first (Fig. 10, interval A). The application of 5 × 10^{-5} M strophanthidin resulted in a slow increase in contracture tension and a
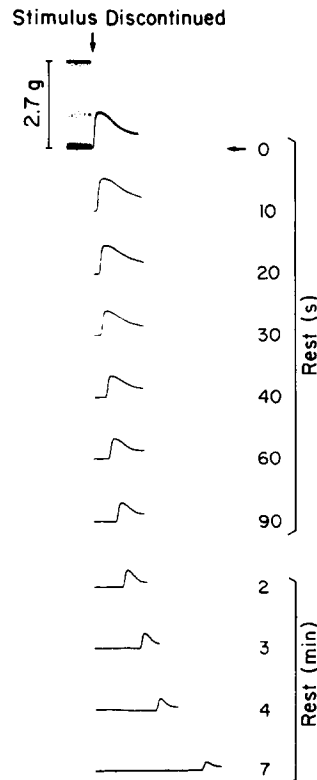

FIGURE 8. Cooling contractures elicited after increasing periods of rest. In the first trace, the muscle was cooled when stimulation at 30°C was discontinued. The cooling solution contained 2.0 mM Na, 142.0 mM choline chloride. In all subsequent traces, the muscle was stimulated to contract (not shown), the stimulus was discontinued, and the muscle was allowed to rest at 30°C for up to 7 min before it was cooled. This shows the marked decline in cooling contracture magnitude with rest. The numbers on the right are the rest durations.

marked increase in the peak-to-peak tension noise amplitude apparent in the recording of the AC-coupled transducer. This is displayed in Fig. 10, interval B. Abrupt cooling resulted in a cooling contracture and a marked suppression of tension noise (Fig. 10, interval C). Rewarming the muscle resulted in a resumption of the noise and a relaxation of the cooling contracture to precooling tension levels. The Fourier spectra of the tension noise during cooling and after rewarming were different (Fig. 11). The rewarmed muscle hibited noise with a periodic (oscillatory) component that, in the example show   had a peak between 2 and

3 Hz. In five experiments, oscillatory components to the noise were measured with the peak frequencies of this value. These values seem to reside within the range of spectral frequencies reported by others (Orchard et al., 1983; Allen et al., 1984). After cooling the muscle, the Fourier spectrum of the noise exhibited no obvious peaks and was very similar to the spectrum obtained from the noise produced by a transducer from which the muscle had been removed (result not shown). Moreover, the application of 10.0 mM caffeine rapidly and reversibly suppressed tension noise, which had been induced by applying 5×10^{-5} M
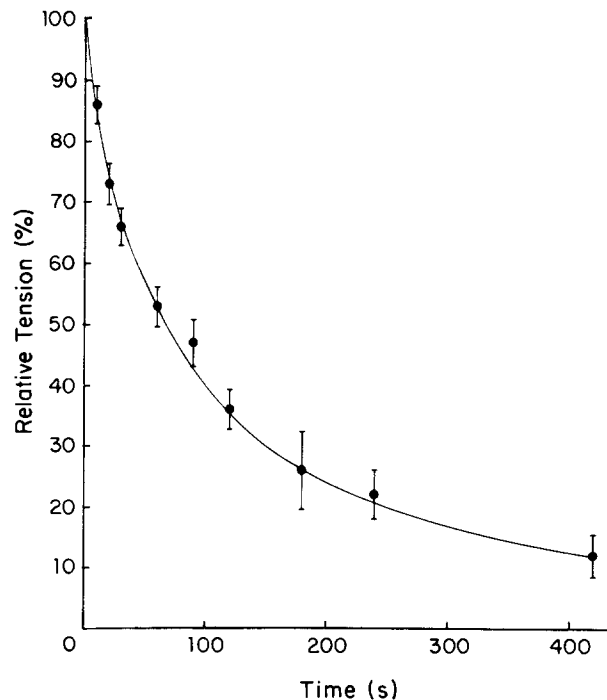

FIGURE 9.   Rest decay of cooling contracture. The relative tension (ordinate) was calculated by dividing the maximum contracture tension obtained immediately after electrical stimulation by the magnitude of the contracture tension after a period of rest. Values are means ± SE (n = 5 muscles).

strophanthidin to the muscle (not shown). The Fourier spectrum of the tension noise of a caffeine-treated muscle resembled that obtained from a cooled muscle.

Since cooling abolishes the microscopic tension oscillations that accompany tonic tension development at 30°C, it is reasonable to assume that the relative magnitude of cooling contractures reflects the relative levels of cytosolic Ca producing them. This conclusion is, of course, valid only over a range of cytosolic Ca values for which the tension-generating mechanism is not saturated.

Measurement of Membrane Potential During Rapid Cooling

It is conceivable that the process or processes that lead to the activation of cooling contracture require electrical depolarization. For instance, a gated entry
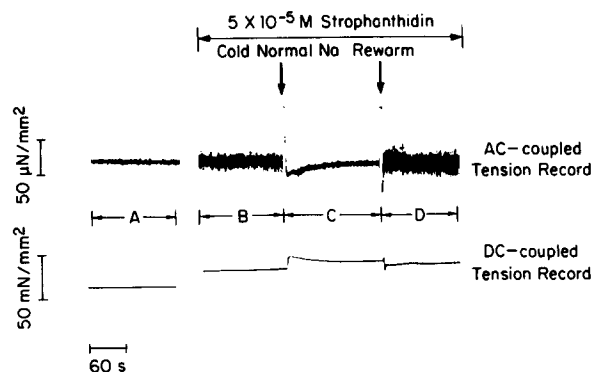
FIGURE 10. The effect of cooling on tension noise induced by the application of 5 × 10⁻⁵ strophanthidin to a resting papillary muscle. Rest tension from the same muscle was recorded with an AC-coupled transducer (top) and a DC-coupled transducer (bottom). Interval *A* exhibits tension recorded before the application of 5 × 10⁻⁵ M strophanthidin. After ~23 min of strophanthidin application, both rest tension (bottom) and peak-to-peak tension noise increased significantly. This is shown in interval *B*. In interval *C*, the muscle was cooled to 0°C. This suppressed tension noise (top) and produced a cooling contracture (bottom). Rewarming the muscle (interval *D*) resulted in a relaxation of the cooling contracture (bottom) and a resumption of tension noise (top).
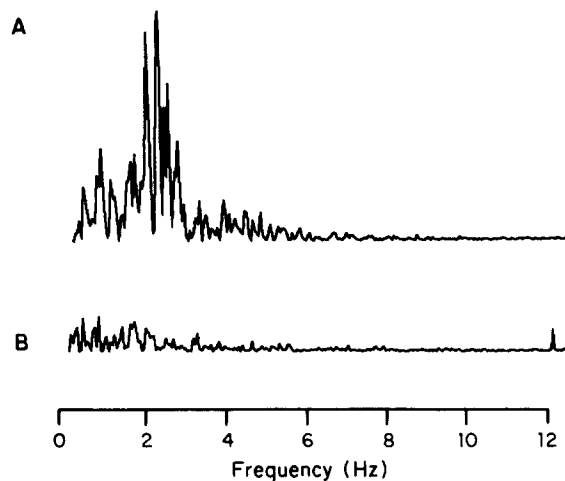

FIGURE 11. (*A*) Fourier spectrum of tension noise produced by application of 5 × 10⁻⁵ M strophanthidin. This spectrum was obtained by sampling muscle tension for 20 s at 25 Hz. The sample was taken during the interval labeled *D* in Fig. 10. (*B*) Second spectrum of tension noise obtained when the muscle was cooled during the interval labeled *C* in Fig. 10. Cooling suppressed the peaks that are clearly evident in *A*.

of Ca may activate contractures. Similarly, a voltage-dependent release of Ca from intracellular stores may be required to activate cooling contractures. I therefore examined the changes in membrane potential that occurred when papillary muscles were cooled abruptly. Contractures tend to displace impaling microelectrodes. Consequently, I took advantage of the fact that contractures were considerably diminished after a period of rest. Single papillary muscles maintained at ~30°C were impaled with KCl microelectrodes. In experiments
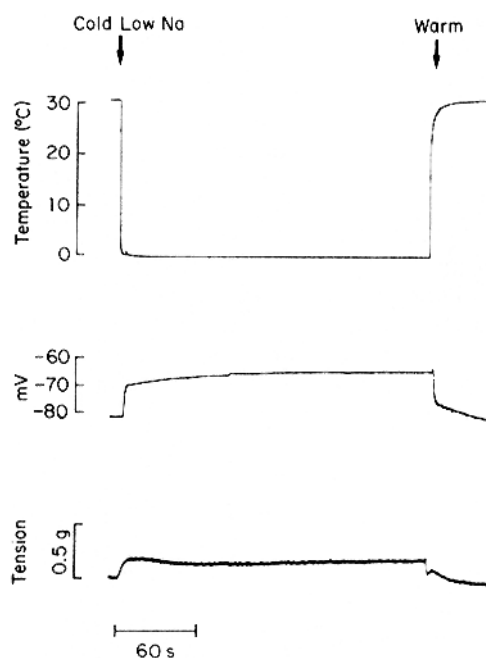

FIGURE 12. A single papillary muscle cooled after prolonged rest. The top panel shows the time course of temperature change of the bath after abrupt cooling (arrow). The middle panel shows the time course and magnitude of change in membrane potential after abrupt cooling and rewarming of the muscle. The bottom panel shows the tension changes accompanying cooling and rewarming.

on seven muscles, the resting membrane potential averaged -79.9 ± 1.0 mV ($n = 15$ observations). Fig. 12 shows the membrane depolarization produced by abruptly cooling a papillary muscle to 1°C. The time course of cooling was measured at the same time as the membrane potential by means of a thermistor placed in the bath. Tension was also measured. Cooling produced a membrane depolarization that exhibited two components: an initial rapid component, followed by a slow component, which in this case was followed for several minutes. Rewarming the muscle resulted in a repolarization that restored the original membrane potential. It should be emphasized that the cooling solution was deficient in Na (2.0 mM), which was replaced with choline chloride (2.0 mM).

Fig. 13 shows simultaneous cooling and depolarization at an increased chart speed. It is clear that the rapid depolarization accompanied the decline in bath

temperature. In 15 measurements on seven muscles, the rapid depolarization (measured seconds after initiating cooling) amounted to 12.9 ± 0.41 mV. After each experiment, the recording electrode was removed from the muscle and placed in the bath. The electrode was then subjected to a temperature drop. This produced an average depolarization of 2.8 ± 5 mV ($n = 5$ observations). The recording system had a differential input with a reference electrode downstream from the muscle. It is possible that after a switch to low temperature, the recording electrode was at a slightly lower temperature than the reference electrode. In this case, the liquid junction potentials at the two electrodes might
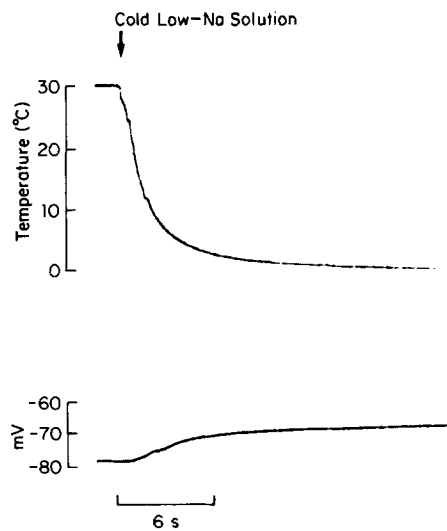

FIGURE 13. Temperature and membrane potential changes after abrupt cooling, depicted on an expanded scale. In the top panel, the muscle was abruptly cooled (in low-Na solution) at the arrow. The curve depicts the ensuing decline in temperature. The bottom panel shows the membrane potential change coincident with abrupt cooling of the muscle surface. These curves were obtained by photographing original, unfiltered records.

have differed. This may explain the modest, but direct, effect of cooling on the recording system.

The initial rapid depolarization that the muscle exhibited after subtracting the electrode effect amounted to 10.1 mV. This is explained if one assumes that these heart cells behave as K electrodes. The direct effect of temperature on the parameter RT/F ensures that the muscle will exhibit a depolarization of ~9.0 mV when it is cooled from 30 to 0°C (membrane potential at 30°C = −80.0 mV). The abrupt membrane depolarization upon cooling is therefore explained (to within 1.0 mV) by a change in thermodynamic constants. The subsequent depolarization amounts to 19.4 ± 2.2 mV after 60 s (11 observations on seven muscles). After subtracting the RT/F temperature effects, this is ~10.0 mV. This depolarization is insufficient to produce a gated entry of Ca (Reuter, 1967). Thus, the process leading to the activation of cooling contractures is unlikely to

be a gated entry of Ca. A number of studies on skeletal and heart muscle indicate that tension development is steeply dependent on voltage (Hodgkin and Horo-wicz, 1960; Beeler and Reuter, 1970). Moreover, this dependence first becomes apparent at about -50 mV. After 30 s of cooling (when tension activation is complete), the membrane depolarized by 16.5 ± 1.2 mV ($n = 13$ observations on seven muscles). Subtracting the RT/F effects, this amounts to ~ 7.0 mV. Thus, cooling contractures are unlikely to be activated by membrane depolarization.

Effect of Caffeine on Cooling Contracture

The results so far suggest that cooling contractures are at least in part activated by Ca that has an intracellular origin. It is reasonable to assume that the SR may play an important role in activating cooling contractures, although depolarization is probably not sufficient to promote a release of SR Ca. If cooling significantly slows Ca pumps, uncompensated Ca leak from the SR will activate tension. Caffeine prevents both frog and rabbit isolated SR from accumulating Ca (Weber and Herz, 1968). The drug should therefore affect the magnitude of cooling contractures if they are activated by SR Ca. This suggestion is strengthened by the fact that other effects of caffeine, e.g., increasing the sensitivity of the contractile elements (Wendt and Stephenson, 1983), would be expected to enhance cooling contractures if they were activated by some process that was otherwise uninfluenced by caffeine. Muscles were rapidly cooled before and after caffeine treatment. The application of 10.0 mM caffeine to muscles stimulated at 0.5 Hz typically resulted in an initial small increase in twitch tension, followed by a decline to a new steady state value that was $75 \pm 5\%$ ($n = 4$ muscles) of the control tension. At this point, the drug was assumed to have taken effect. The results depicted in Fig. 14 are typical of those obtained from five other muscles. Cooling contractures were almost completely suppressed in muscles pretreated with caffeine. For example, ~ 20 s after cooling, significant contracture was developed in untreated muscles, but in the same length of time, an untreated muscle developed $\sim 2.5\%$ of this tension.

Muscles were also cooled in solutions of differing composition and the results were examined over longer periods of time (Fig. 15). Caffeine-treated muscles (Fig. 15) responded to abrupt cooling with a very slight and slowly developing increase in tension, which is apparent in recordings made at lower chart speed. This slow creep in tension was identical in muscles cooled in solutions deficient in Na (2.0 mM) and in solutions containing normal Na (144.0 mM). Consequently, this slow creep in tension is unlikely to represent a slowly developing Na-withdrawal contracture. During cooling, a passive transsarcolemmal Ca leak exists that will eventually lead to a contracture development. In the presence of caffeine, this might be more evident because the contractile elements are thought to be sensitized by the drug.

The marked suppression of cooling contractures by pretreatment of muscles with caffeine suggests that the SR is involved in their activation. This further suggests that in the presence of caffeine, the role of the SR in producing twitches is either diminished or absent. It is therefore possible that twitches elicited at room temperature in the presence of 10.0 mM caffeine are activated solely by
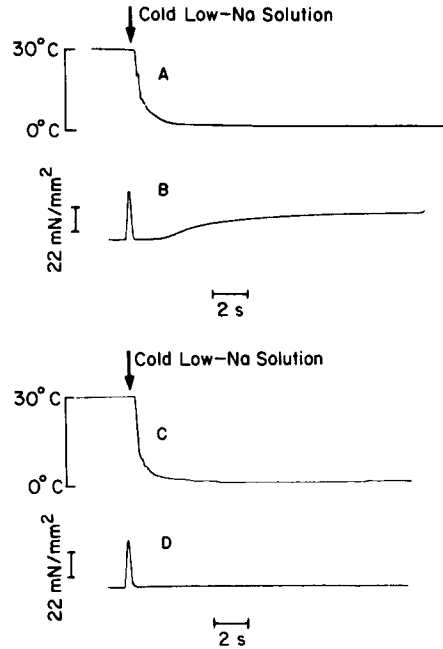
FIGURE 14. Effect of 10.0 mM caffeine on cooling contractures. Curves *A* and *C* indicate the time course with which the muscle surface cooled. Curve *B* depicts the time course with which the muscle developed contracture tension after cooling. Curve *D* depicts the effect of cooling the same muscle after pretreatment with 10.0 mM caffeine.
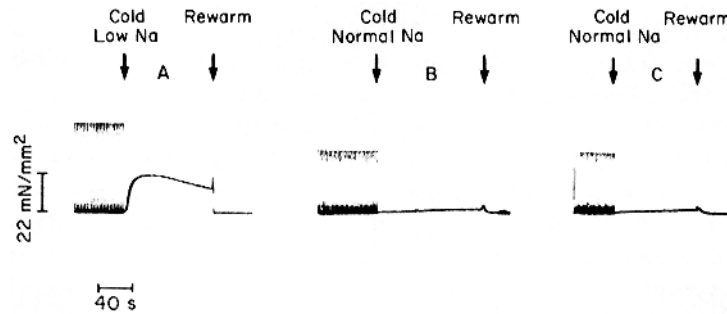
FIGURE 15. Effect of cooling a caffeine-treated muscle, displayed at a slower chart speed. (*A*) Effect of cooling and rewarming an untreated muscle. A typical cooling contracture results. (*B* and *C*) Result of cooling the same muscle after caffeine pretreatment (10.0 mM). In *B*, the muscle was cooled in Na-deficient (2.0 mM Na, 142.0 mM choline chloride) solution. (*C*) After rewarming and recovery to stable twitch levels (in 10.0 mM caffeine), the muscle was again cooled in a standard Tyrode's solution (144 mM Na). In both cases, a slow creep in tension is apparent. The magnitude of this creep appears similar, irrespective of the composition of the cooling solution. The arrows indicate when abrupt cooling and rewarming took place.

transsarcolemmal Ca influx (see Discussion). In the presence of caffeine, the SR may make no significant contribution to tension development.

Factors Influencing the Decay of Cooling Contractures

Cooling contractures appear to be activated by SR Ca. Rest reduces the magnitude of these contractures. If it is first assumed that diminished contractures reflect diminished SR Ca content, this finding is explained by a simple hypothesis. During rest, Ca first leaks from the SR into the cytosol. It is then pumped to the cell exterior. In support of this hypothesis, Kitazawa (1984) has recently demonstrated that Ca leaks from the SR of skinned cardiac muscle with a time constant of 40–60 s. The inhibition of SL Ca transport (and therefore the means of net Ca extrusion) might prevent a loss of SR Ca. This hypothesis implies that SL Ca transport influences SR Ca content and contractility.

The transsarcolemmal Na/Ca exchange is thought to pump Ca out of heart cells at the expense of an inwardly directed Na gradient. If the gradient is collapsed, Ca pumping by Na/Ca exchange will cease and may even be reversed (Mullins, 1981; Chapman et al., 1983). In the absence of extracellular Na (and therefore reduced or reversed transsarcolemmal Ca movement), the loss of SR Ca should either be reduced or reversed. The rest decay of cooling contracture should therefore be affected by extracellular Na.

Fig. 16A shows the effect of reducing extracellular Na on the rest decay of cooling contractures. First, a typical control decay curve was obtained in 144.0 mM Na (Fig. 16A). The muscle was then used to examine the effect of low Na on the decay of cooling contracture. Upon discontinuing stimulation, the superfusing solution was exchanged for one containing 2.0 mM Na and 142.0 mM choline chloride, which was used as a replacement ion. After a suitable initial rest interval, a cooling contracture was elicited. The muscle was then superfused with normal Tyrode's solution and reequilibrated. The procedure was repeated with a longer rest interval. The longest rest interval in 2.0 mM Na was 10 min. For rest intervals longer than this, Na was readmitted. This change is marked by the arrows in Fig. 16, A and B. Muscles rested and superfused with 2.0 mM Na showed an initial decay in contracture magnitude (Fig. 16). This was followed by a large increase in contracture magnitude, which was in excess of control values (all contractures are expressed relative to the zero-time contracture). The contractures appeared to reach a plateau after 5 min, and a slow decline was observed in some experiments. The readmission of normal Na after 10 min of rest induced an immediate rest decay of contractures similar to the control rest decay (curve A). Either the absence of extracellular Na or the presence of choline chloride prevented and to some extent reversed the rest decay of cooling contractures. A similar experiment (Fig. 16B), using Li as the replacement ion, produced the same result, which suggests that the result is specifically produced by the absence of extracellular Na rather than by the presence of choline chloride.

This result is consistent with the notion that intracellular Ca stores are not depleted when Ca extrusion via Na/Ca exchange is prevented. Moreover, the observed increase in contracture tension above control values can be attributed to replenishment of intracellular stores. This may result from uncompensated

Ca leak across the sarcolemma or reversed Na/Ca exchange in the presence of low extracellular Na.

The initial decay of cooling contracture upon superfusion with low Na is also consistent with the foregoing hypothesis. The decline in extracellular Na is not immediate, because of diffusion delays. The effect of low Na will be apparent when extracellular Na has declined to a value that causes a reversal of Na/Ca
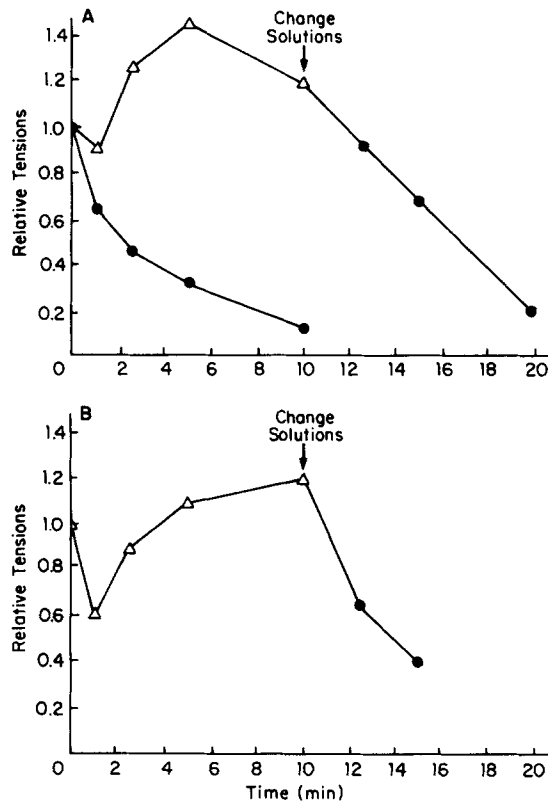

FIGURE 16. (*A*) Typical rest curve in 144.0 mM extracellular Na (●). Effect of 2.0 mM Na on rest decay of cooling contracture (△). Returning 144.0 mM Na to the Tyrode's solution caused abrupt rest decay of cooling contractures. Choline chloride was used to replace Na. (*B*) Identical experiment, except that Li was used to replace Na.

exchange. If it is assumed that the stoichiometric coefficient of the Na/Ca exchange is 3.0 (Bridge and Bassingthwaighte, 1983; Reeves and Hale, 1984), one can use the following equation to calculate the concentration of extracellular Na at which the exchange reverses:

$$[Na]_o = \left([Na]_i^3 \, \frac{[Ca]_o}{[Ca]_i} \, e^{EF/RT} \right)^{1/3} .$$

Assuming that, at rest (30°C), $E = -80.0$ mV, $Na_i = 10.0$ mM, $Ca_o = 2.7$ mM, and $Ca_i = 10^{-7}$ M, it follows that the exchange will reverse when $[Na]_o = 108$ mM. Therefore, Na/Ca exchange will continue to extrude Ca until extracellular Na has reached 108 mM. One can obtain a rough idea of when this will occur by calculating the time required to reduce extracellular Na to this value upon instantaneously dropping the bath Na from 142 to 2.0 mM. The following solution to the diffusion equation in cylindrical coordinate is given by Crank (1975) as

$$\frac{M_t}{M_\infty} = 1 - 4 \sum_{n-1}^{\infty} \frac{\exp\left(\frac{-Dt}{a^2} \alpha_n^2\right)}{\alpha_n^2},$$

where α_n is the nth root of $J_0(\alpha_n) = 0$, D is a radial diffusion coefficient, a is the fiber radius, and M_t and M_∞ are the quantities of Na/Ca leaving the fiber at time t and at infinite time, respectively. The ratio M_t/M_∞ is the fractional exchange. The mean extracellular concentration at time t can be calculated from the value of M_t/M_∞. The value of D must be corrected for tortuosity. The tortuosity factor λ for heart muscle is 1.38 (Page and Bernstein, 1964). The corrected value of D is 7.1×10^{-6} cm^2/s. (The value of D obtained for Na in H_2O is tabulated for 25°C and the small correction to 30°C has not been made.) Using a value of 1.0 mm for the muscle diameter, one can calculate that 8–9 s would be required to reduce the mean extracellular Na concentration to 108 mM when Na/Ca exchange is assumed to reverse direction. After 23 s, the extracellular Na concentration would be 73 mM. This would reverse Na/Ca exchange and cause Ca entry. Although the time of inflection was not carefully examined in this study, it is clear from the foregoing calculation that a delay in the effect of reducing extracellular Na is expected. The initial inflection is certainly more pronounced in Fig. 16B than in 16A. This might be explained by the fact that the muscle used to produce Fig. 16A has a smaller diameter (1.0 mm) than that used in Fig. 16B (1.1 mm).

The rest decay in cooling contractures is accelerated in the presence of caffeine (Fig. 17). First, a control curve reflecting the rest decay of cooling contracture was obtained in the manner already described. For reasons that will be apparent, points were not obtained for the first 2 min of rest. After 2 min of rest, contracture tension declined to a value of ~50% of the initial contracture tension. In a second experiment (using the same muscle), 10.0 mM caffeine was applied during the rest period. 2 min was allowed before applying the cold flush to increase the chances that the caffeine would take effect. In the presence of caffeine, 2 min of rest reduced the magnitude of cooling contractures to ~19% of the initial contracture magnitude (Fig. 17). Caffeine therefore accelerates the rest decay of cooling contractures. After 4 min rest in 10.0 mM caffeine, cooling contractures were reduced to 8% of the initial contracture tension. In contrast, 4 min rest in the absence of caffeine reduced contracture tension to 25% of the initial contracture tension. This is different from the effect of 4 min rest in the absence of caffeine. Ca is hypothesized to leak from the SR into the cytosol, from which it is pumped to the exterior. The acceleration of rest decay by caffeine is

best explained by supposing that the drug accelerates the SR Ca leak, with the result that Ca is more rapidly extruded to the cell exterior. Thus, cooling contractures decline more rapidly in the presence of caffeine than in its absence. Similar explanations for the accelerated decline of potentiated contractions after caffeine application have been suggested by Bass (1976).

Rest-induced Changes in Intracellular Ca Content

The results and hypotheses presented so far suggest that small quantities of Ca are lost from heart cells during rest. To measure these losses, pairs of papillary
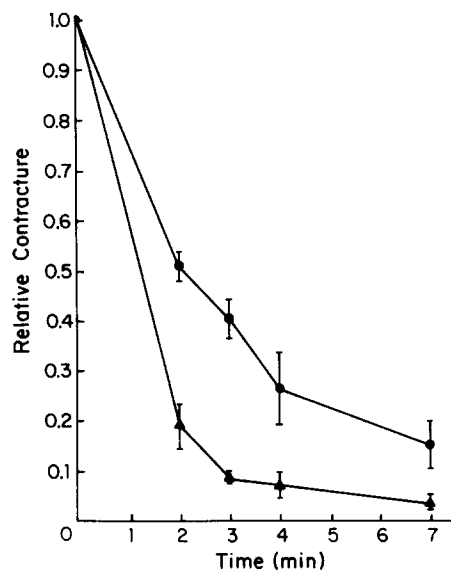

FIGURE 17.   Effect of 10.0 mM caffeine on rest decay of cooling contractures (▲) contrasted with rest decay in the absence of caffeine (●). Values are means ± SE (n = 5 muscles).

muscles from the same animal were analyzed for apparent intracellular Ca content. Each one of a pair was first mounted in adjacent and identical muscle baths. The muscles were then superfused with a modified HEPES-buffered Tyrode's solution containing the extracellular marker KCoEDTA (Bridge et al., 1982). The muscles were stimulated at 30°C and 0.5 Hz for at least 1 h in this solution to assure complete equilibration of the marker. After equilibration, one muscle was rested for 20 min. The rested muscle was chosen randomly by tossing a coin at the beginning of the experiment (Snedecor and Cochran, 1974).

The results from 14 pairs of papillary muscles are displayed in Table I. The apparent intracellular Ca content for the 14 control (stimulated muscles) was 4.62 ± 0.46 mmol/kg dry wt. The experimental rested muscles contained 3.32 ± 0.45 mmol/kg dry wt. The mean difference between pairs was 1.29 ± 0.38 mmol/kg dry wt. A two-tailed paired t test was applied to these differences, which were found to be highly statistically significant ($P \leq 0.005$). Thus, a mean quantity of 1.29 ± 0.38 mmol/kg dry wt was lost from the rested muscles. Muscle

dry weight is ~20% of the wet weight. Therefore, the net loss of Ca by the rested muscle amounted to ~258 μmol/kg wet wt. If the rest decay of cooling contractures is to be explained by net leak of Ca from the SR and subsequently across the SL membrane to the extracellular space, it is clearly desirable to measure sufficient Ca loss. Fabiato (1983) has calculated that 142 μmol total Ca/ liter of cell H_2O accessible to Ca is required to produce 70% of maximum possible tension. The loss of Ca reported in this study is clearly sufficient (assuming that it comes from the SR) to produce the observed rest decay of

TABLE I

Intracellular Ca Content in Paired Muscles

Experiment	Stimulated	Rested	Δ
	mmol/kg dry wt	*mmol/kg dry wt*	*mmol/kg dry wt*
1	3.30	2.07	1.23
2	2.38	2.76	−0.38
3	2.60	1.36	1.24
4	3.31	3.04	0.27
5	4.04	4.48	−0.44
6	5.19	4.77	0.42
7	7.10	4.48	2.62
8	3.70	4.00	−0.30
9	5.00	2.58	2.42
10	2.86	1.16	1.70
11	5.62	4.49	1.13
12	7.80	7.17	0.63
13	5.52	1.25	4.27
14	6.22	2.92	3.30
$\bar{X}$	4.62	3.32	1.29
SD	1.71	1.68	1.43
SE	0.46	0.45	0.38

Apparent intracellular Ca content was measured in pairs of muscles from the same animal. Pairs of muscles were stimulated at the same rate and temperature (30°C). One member of the pair was allowed to rest for 20 min at 30°C. Both pairs were then analyzed for intracellular Ca. The difference in Ca content between the stimulated control and rested experimental muscle reflects Ca lost from the cell during rest.

cooling contractures. Solaro and Briggs (1974) have proposed that the SR of mammalian heart muscle can sequester 170 nmol/g wet muscle when the ambient Ca is 10^{-6} M. The quantities measured in this study are in excess of those measured by Solaro and Briggs, but not markedly so. This further supports the conclusion that rested heart muscle loses sufficient Ca to explain the rest decay of tension.

Muscles rested for 20 min and stimulated at 30 beats/min require 91.8 ± 11.9 ($n = 4$) beats to restore tension to pre-rest levels. If it is assumed that this is the number of beats required to restore the intracellular Ca that is lost during rest, one can calculate that a minimum of 2.8 μmol/kg wet wt Ca enters the cell during each beat. The estimate is a minimum because it is assumed that an equal quantity of Ca enters during each beat. However, if the Ca gain that results from

electrical stimulation after a period of rest approaches an asymptote, the net Ca gain will be greater initially and will decline as the asymptote is approached. It should also be realized that this estimate is only applicable to non–steady state twitches.

DISCUSSION

Activation of Cooling Contractures

The following results suggest that cooling contractures are activated by SR Ca. First, the contracture magnitude is reduced if the contracture is activated after a period of rest. In the absence of changes in extracellular Ca, this result is most easily explained by supposing that some intracellular store of activating Ca depletes with rest. Similar suggestions have been advanced to explain rest decay in other types of contraction (e.g., Allen et al., 1976; Bass, 1976). Lowering extracellular Na to 2.0 mM during rest is likely to cause net Ca entry (Chapman, 1974; Chapman et al., 1983; Allen et al., 1983). It also enlarges subsequent cooling contractures and prevents their rapid decay (Fig. 16, A and B). It is as if intracellular stores of Ca had been replenished and prevented from depleting by reduced extracellular Na. Caffeine, which is thought to release Ca from the SR, suppresses cooling contractures reversibly. Recently, Kurihara and Sakai (1985) observed similar cooling contractures in guinea pig papillary muscle.

If the SR is the source of activating Ca, it is necessary to explain the slow activation. It is possible that some voltage-dependent release of Ca from the SR or gated entry of Ca might rapidly elevate cytosolic Ca with ensuing slow tension development. The observed extent of membrane depolarization makes this unlikely. Although muscle cooling is complete within 2.5–3.0 s, maximum tension required 20–30 s to develop. A simple explanation is that rapid cooling partially or completely immobilizes both SR (Salama and Scarpa, 1983) and SL Ca transport. Ca that leaks into the cytosol from the SR will not be removed rapidly and will activate tension. The calculations presented in the Results support this explanation. The conclusion is further supported by noise analysis of the tension data. The application of 5×10^{-5} M strophanthidin to resting muscle dramatically increases tension noise measured at high gain. Spectral analysis of this noise revealed a periodic component between 2 and 3 Hz. This periodic component has been attributed to the oscillation of Ca between the cytosol and SR (Orchard et al., 1983; Allen et al., 1984). It is reasonable that any oscillation between the SR and cytosol requires the participation of the Ca pump. Immobilization of the Ca pump would be expected to interfere with or even eliminate the oscillations induced by strophanthidin. Cooling, which should immobilize the Ca pump, also eliminates tension oscillation as expected.

If SL Ca pumps are immobilized by cooling, there will be an uncompensated leak of Ca from the extracellular space into the cells. A heart muscle cell of 5 μM radius will have a surface-to-volume ratio of 0.4×10^4/cm. Assuming that Bianchi's (1961) estimate of Ca influx into resting muscle (9.4×10^{14}/cm$^2 \cdot$s) applies to heart, I calculate that the concentration of cell Ca will change by ~1.0 μmol as a result of 20 s of sustained transsarcolemmal Ca leak. This is not

sufficient to activate tension. It is therefore probable that the principal source of activating Ca is the SR.

The foregoing results and calculations suggest that, upon cooling, Ca might leak from the SR in sufficient quantities to activate contraction. There are other ways that tension might be activated. For instance, lowering the temperature might increase the sensitivity of the contractile elements. Stephenson and Williams (1981) observed that a change in temperature from 5 to 25°C rendered fast-twitch rat skeletal fibers less sensitive to Ca by a factor of 2. However, the effect of temperature on force generation appears to be complex. Godt and Lindley (1982), working on mechanically skinned frog semitendinosus muscle, demonstrated that the pCa value required for 50% maximal force development was inversely proportional to temperature. However, maximal force increased with temperature. These results present a paradox. Similar results (over less extreme ranges of temperature) have recently been reported by Fabiato (1985) for skinned canine Purkinje fiber. The results of Godt and Lindley (1982) may provide some insight into the activation of cooling contractures reported in this article. These authors obtained two pCa vs. tension relationships (their Fig. 2), one for a muscle maintained at 4°C and the other for a muscle maintained at 22°C. Force was expressed relative to the maximum obtained at 4°C. Tension saturated at pCa's of ~6 (4°C) and ~5 (22°C). The two relationships (which differed) intersected once, at a pCa of ~6.25. Below the concentration at which intersection occurred, the low-temperature curve showed greater sensitivity to Ca than the high-temperature curve. The converse was true above the intersection. Godt and Lindley suggest that abrupt temperature changes shift the muscle contractile machinery from one relationship to the other with predictable consequences.

Fig. 5 shows that abruptly heating a muscle at the peak of low-temperature activation causes an abrupt increase in tension. Similar results were obtained by Godt and Lindley (1982) on skinned fibers. They were interpreted by supposing that ambient Ca is above the intersection value. When the muscle is heated, it shifts to the high-temperature relationship and therefore produces more tension. The behavior of rabbit papillary muscle is consistent with the results and proposals of Godt and Lindley (1982). Low temperature seems to suppress maximum tension development and is unlikely to activate cooling contractures.

The experiments conducted with caffeine strengthen the view that cooling contractures are principally activated by SR Ca. It is clear from Fig. 15 that in the presence of 10.0 mM caffeine, electrical stimulation produces twitches. However, cooling contractures are almost completely suppressed in the presence of the drug. The evidence that caffeine leads to a loss of SR Ca is compelling (Weber and Herz, 1968). Fabiato and Fabiato (1972, 1973) have concluded that caffeine blocks the uptake of Ca into the SR, so that its Ca stores are depleted. Hess and Weir (1984) have shown that high doses of caffeine (4.0–10.0 mM) virtually eliminate both an initial (L_1) and a plateau-like (L_2) component of the aqueorin transient. While there has been considerable debate about the precise origin of L_1 and L_2, Hess and Weir speculate that a possible source of Ca^{2+} for L_1 is the SR. In view of these data, caffeine might be expected to reduce or

abolish cooling contractures if they are activated by SR Ca. However, caffeine does not specifically release SR Ca. Fabiato and Fabiato (1976), Endo and Kitazawa (1978), Fabiato (1981), and Wendt and Stephenson (1983) have all demonstrated that caffeine increases the Ca sensitivity of skinned cardiac fibers. If cooling contractures are activated by Ca from some source other than the SR, then one would expect caffeine to increase rather than suppress tension during the cooling contracture.

The presence of a slow and small increase in contracture tension when caffeine-treated muscles are cooled is unlikely to be an Na-withdrawal contracture, since it is also present when the cooling solution is not deficient in Na. Passive leak of Ca into cooled muscles is expected because all transsarcolemmal Ca pumping is probably immobilized. Presumably, the SR is no longer available to buffer incoming Ca. Moreover, the sensitivity of the contractile elements is increased in caffeine. The resulting slow creep in contracture tension is therefore not surprising. Given these conclusions, the twitches obtained in the presence of caffeine are activated by Ca from some source other than the SR. Caffeine may increase the sensitivity of the contractile elements so that transsarcolemmal Ca influx is sufficient to activate tension.

It is not clear why cooling contractures are transient. The secondary tension decline is probably not due to Ca transport out of the cell as a result of incomplete immobilization of Ca transport. The cooling solution was deficient in Na (2.0 mM), so that net Ca entry, rather than exit, would be expected. Morever, cooling contracture is uninfluenced by the level of Na in the cooling solution. Salama and Scarpa (1983) show that isolated SR from rabbit skeletal muscle ceases to sequester Ca below 5°C. In heart muscle, some residual sequestering of Ca may occur at low temperature. It is also possible that other sinks, e.g., mitochondria or slow Ca buffering (Robertson et al., 1981), might play a role in producing extremely slow Ca transients. Other mechanisms, which might slowly inactivate tension, include a thermoelastic effect, slow pH changes, and a direct effect of cooling on the contractile machinery. The presence of slow tension inactivation in the cold does not alter the conclusions drawn from this study.

Decay of Cooling Contractures

The rest decay of cooling contractures is most easily explained by assuming that Ca continually leaks from the SR into the cytosol and is then pumped across the SL to the cell exterior. Endo and Kitazawa (1978) and Kitazawa (1984) have shown that in saponin-skinned guinea pig ventricle, the SR leaks Ca (at a greater rate than skeletal muscle SR). Moreover, Kitazawa's recent results indicate a $t_{1/2}$ for the leak of 40–60 s. This is close to the $t_{1/2}$ of 70 s for rest decay of cooling contractures measured in this study. This comparison might indicate that the rate of SR Ca leak largely determines the rate of rest decay in intact preparations. A net loss of cellular Ca will occur only if Ca efflux exceeds influx during rest. There are two pathways for Ca extrusion. One is an ATP-driven Ca pump that is thought to possess a high affinity and low capacity. The other is Na/Ca exchange, a low-affinity, high-capacity system that is driven by the electrochemical Na gradient (Caroni and Carafoli, 1980). There is little information on the

relative importance of Na/Ca exchange and the ATP-driven Ca pump in compensating resting Ca leak. This hinders interpretation of the effect of low Na on rest decay of cooling contractures. If the inward Na gradient is partially collapsed by removing extracellular Na, the Ca pump (unlike Na/Ca exchange) should remain unaffected. Biochemical data (Caroni and Carafoli, 1981) suggest that the K_m for the Ca pump is ~0.3 μM, whereas the K_m for Na/Ca exchange is of the order of 1.5 μM. Since cytosolic Ca in resting heart muscle is of the order of 10^{-7} M, it is likely that the Ca pump plays a dominant role in compensating passive leak in resting heart.

A sudden reduction of extracellular Na should reverse Na/Ca exchange (Mullins, 1981) and cause net entry of Ca via the exchange. Ca entry is now expected to occur by two pathways, passive leak and reversed Na/Ca exchange. After a brief diffusion delay, cooling contractures increase, which indicates that the SR is filling. In the virtual absence of extracellular Na, cooling contractures show very little sign of rest decay. There are two plausible explanations for this. As already explained, lowering extracellular Na probably reverses Na/Ca exchange and initially promotes Ca entry. As intracellular Na declines (because of the combined effects of reverse Na/Ca exchange and low extracellular Na), Na/Ca exchange may slow or cease completely. Under these circumstances, the Ca pump may not be capable of discharging (in the face of continued electrodiffusive Ca leak) the Ca accumulated by reverse Na/Ca exchange. This implies that the rest decay of cooling contractures normally requires both the presence of Na/Ca exchange and the Ca pump. An alternative explanation is that reverse Na/Ca exchange causes a gain in Ca. A simultaneous decline in intracellular Na also occurs. However, instead of completely immobilizing Na/Ca exchange, some residual reverse Na/Ca exchange persists, despite the reduced intracellular Na. Therefore, to remove Ca from the cell, the Ca pump must not only compensate passive Ca leak, but must also extrude Ca that is continually brought into the cell via Na/Ca exchange. It is unlikely that these possibilities can be distinguished without specific inhibitors of the Ca pump and Na/Ca exchange. Whatever the details of the mechanism of rest decay of cooling contractures, the results strongly support the notion that the decay depends upon SL Ca transport. Caffeine accelerates the rest decay of cooling contractures. The effect of the drug can be explained by the foregoing scheme it if is assumed that caffeine accelerates the loss of Ca from the SR, which is in turn pumped to the exterior at an increased rate (Jundt et al., 1975; Bass, 1976).

To measure the anticipated loss in intracellular Ca produced by rest, I used pairs of muscles from the same heart. The measurement of Ca loss was based on statistical inference from 14 pairs of papillary muscles. A value for apparent intracellular Ca was deduced by first measuring tissue Ca and then subtracting the Ca distributed in the extracellular water (as measured by the distribution of KCoEDTA [Bridge et al., 1982]). The difference in apparent intracellular Ca content between appropriately treated pairs was then calculated. The need for pairing muscles to measure changes in intracellular Ca content can be appreciated by considering the various assumptions and difficulties associated with the measurement of analytical intracellular Ca in a multicellular preparation. One must

first measure extracellular Ca. This requires knowledge of the amount of Ca bound to the external surface of cells, the amount bound to extracellular substances, and finally the amount of Ca distributed in the extracellular water. The distribution of extracellular water is probably reliably estimated by the distribution of KCoEDTA (Bridge et al., 1982). However, CoEDTA$^-$ and Ca^{2+} are unlikely to share the same adsorption isotherm in the extracellular space. These are, for instance, negatively charged substances in the extracellular space (Comper and Laurent, 1978) that could bind significant quantities of Ca, whereas the results of Bridge et al. (1982) indicate that CoEDTA$^-$ is not absorbed in the extracellular space. This means that some extracellular Ca will be computed as intracellular Ca. It is clear that a correct estimate of intracellular Ca is impossible using this approach. In this study, estimates of the absolute value of apparent intracellular Ca are not assigned great significance. The quantity of significance is the change in apparent intracellular Ca resulting from a particular experimental intervention—in this case, rendering a muscle quiescent. The differences in apparent intracellular Ca content between pairs of muscles are attributed to differences in the ways in which each member of a pair has been treated. When one takes the difference between pairs, errors that are common to each one of the pair will be eliminated if inferences are based upon a statistic that is calculated from a suitable sample of pairs. Errors common to both muscles must of course be randomized (in this case by tossing a coin) before the experimental intervention. This approach to the analysis of changes in intracellular Ca content hinges upon the following assumptions. (*a*) There are, before the experimental interventions, no statistically significant differences in Ca content between pairs. (*b*) Extracellular Ca is not detected by the extracellular marker, i.e., that which is bound is, when referenced to dry weight, identical in both pairs of muscles. Thus, extracellular bound Ca must be the same in quiescent and stimulated muscles. (*c*) The volume of extracellular H$_2$O that excludes KCoEDTA should be small.

The available data (not shown) suggest that assumption *a* is justified. Pairs of identically treated (rested) and randomized muscles analyzed for apparent intracellular Ca indicated that there is no significant difference in the apparent intracellular Ca content between pairs. With regard to assumption *b*, there is little information. The quantity of Ca bound to the external SL might be quite large, i.e., ~700 μmol/kg wet wt (Bers and Langer, 1979). As already mentioned, structures in the extracellular space other than the external SL may also bind Ca. However, unless the stimulation frequency affects extracellular (including SL surface) Ca binding, the estimates of Ca loss with quiescence will remain unchanged. There is no a priori reason to suppose that significant Ca is lost from surface binding sites when a muscle becomes quiescent.

If CoEDTA$^-$ is excluded from a significant volume of extracellular H$_2$O, then the Ca residing in that water would be computed as intracellular. Were this volume of water to change significantly with stimulation, the conclusions about the change of intracellular Ca with rest would be incorrect. The exclusion of CoEDTA$^-$ from the extracellular space might be based on electrical charge, in view of the negatively charged carboxyl and sulfate groups associated with the

ground substance (Haljamae et al., 1974). However, the similarity of distribution of electroneutral sucrose and CoEDTA⁻ in the extracellular space led to the conclusion that charge exclusion effects are negligible (Bridge et al., 1982). Since steric exclusion usually occurs in narrow regions like the T-tubules, it is unlikely to play a significant role in these estimations of extracellular space. This is because the T-tubules are at least 1,000 Å in diameter (McNutt and Fawcett, 1974), far larger than the diameter of a molecule like CoEDTA⁻. Therefore, extracellular water and the extracellular Ca that it contains are probably estimated correctly by KCoEDTA.

With the randomized paired-muscle approach, errors common to both muscles are rejected. The disadvantages of this approach are the destructive nature of the assay and the fact that the precision of the method only increases as the square of the number of trials. The latter disadvantage is particularly serious because the method is both difficult and tedious, so that obtaining sufficient pairs for a satisfactory sample may be prohibitive for some measurements.

The result of analyzing 14 pairs of muscles is that 20 min of rest (control muscle stimulated at 0.5 Hz) produces a loss of Ca that amounts to 1.29 ± 0.38 mmol/kg dry wt. This loss is statistically significant ($P \leq 0.005$) and amounts to ~258 μmol/kg wet wt. Since 14 stimulated muscles contained 4.62 ± 0.49 mmol/kg dry wt or ~924 μmol/kg wet wt, it appears that rest induces a loss of ~28% of the cell Ca. It is impossible to say how much of this Ca is lost from the SR or other intracellular sinks. However, Solaro and Briggs (1974) estimated that under circumstances where the ambient Ca was 10^{-6} M, isolated SR vesicles contained 170 μmol Ca/kg wet heart muscle. Dani et al. (1979) concluded from their study of isolated myocytes that SR-related Ca sequestration amounted to between 100 and 300 μmol/kg wet tissue. Hunter et al. (1981) have measured rapidly exchangeable cellular Ca^{2+} in perfused beating rat heart. After labeling the heart with isotope, they immobilized exchange by cooling. During the cooling period, extracellular Ca was washed away. Reperfusing the heart at 37 °C released 125 μmol of exchangeable cellular ⁴⁵Ca/kg wet heart. The similarity between these various measurements and the losses of SR Ca reported in this study lead to the tentative conclusion that the rest-induced loss of Ca represents Ca that was predominantly stored in the SR. Some of this Ca may have some other origin, e.g., the mitochondria. However, sufficient Ca is lost to explain the rest decay of cooling contractures.

A rested muscle requires on average ~90 beats to restore twitch tension (to pre-rest levels) and therefore any Ca lost during rest. This suggests that a minimum of 2.8 μmol Ca/kg wet wt enters a heart cell during a single beat. The estimate is a minimum because the gain of Ca is likely to be asymptotic. Initial values of Ca gain are therefore required to estimate the Ca gain per beat. It is not possible to say by how much this minimum value underestimates the Ca entry per beat. However, recent estimates of Ca entry during the first 100 ms of Ca current in isolated bovine ventricular myocytes result in a total Ca increase of 25 μM (Isenberg, 1982). This would correspond to ~12.5 μmol/kg wet wt for intact tissue, assuming extracellular H_2O is about half the tissue water. Bers (1983) has measured transient depletions of extracellular Ca during the action potential. He has inferred that 11 μmol/kg wet wt of Ca enters at this time,

although the physiologically low extracellular Ca used in his experiments might lead to an underestimation. My results agree to within one order of magnitude with those of Bers and Isenberg. This is encouraging in view of the fundamentally different approaches and conditions.

Cooling contractures appear to provide a relative index of the availability of SR Ca for contraction. They also represent the relative degree of replenishment of the SR if it is assumed that the availability of the SR Ca for contraction reflects the magnitude of SR Ca content. This interpretation of the data also requires the assumption that the relative magnitude of cooling contractures reflects the relative level of cytosolic free Ca activating the cooling contractures. It has been proposed that, in the presence of Ca oscillation, tonic tension of any sort might not provide a useful reflection of the level of Ca that activates the tension. In the presence of Ca oscillations, it is meaningless to assign a discrete Ca value to a particular tension (Allen et al., 1984). The abolition of periodic tension noise and therefore Ca oscillation by cooling suggests that a particular magnitude of cooling contracture may be associated with a discrete Ca level. The advantage of cooling contractures over twitches as a possible index of intracellular store replenishment is that contractures appear to be activated solely by intracellular stores. Twitches may be activated in part by extracellular Ca. An additional advantage is that cooling contractures may be elicited under circumstances (e.g., low Na^+, high K^+; unpublished data) in which it is difficult to elicit twitches. They may therefore be valuable in assessing the extent of intracellular store replenishment under a wide variety of circumstances.

Implications for the Regulation of Contractility

The data presented in this study lead to the inference that SL Ca transport can influence the availability and probably the extent of SR Ca replenishment. During a series of identical twitches, the quantity of intracellular Ca averaged over one complete contraction-relaxation cycle must remain unchanged. That is, if the cellular Ca content is to remain unchanged, the time-averaged Ca influx must be matched by the time-averaged Ca efflux. Anything that changes this balance will cause a change in the intracellular Ca content. For instance, if Ca efflux declines somewhat and influx remains unchanged, the intracellular Ca content will increase. If some of this Ca increase appears in the SR, then an increase in contractility will occur. Similarly, a decline in the time-averaged Ca efflux with unchanged influx will also produce a rise of intracellular Ca. Thus, a small imbalance in SL Ca transport (both passive and active) will be accumulated by the cell and could be stored in the SR.

An influence of SL Ca transport on SR Ca content of this type might provide an explanation of the positive force-frequency relationships that are observed in mammalian heart muscle (Koch-Weser and Blinks, 1963). Considering a fixed period of time, during which successive twitches increase in magnitude as a result of doubling the beat frequency, the time-averaged influx of Ca via the slow inward current will be greater during this interval than during a similar interval of time preceding the frequency increase. During this interval, the membrane will also spend more time depolarized since the quantity $\int E_m \cdot dt$ for this interval will increase. It is assumed for the sake of illustration that the action potential

duration does not change significantly. If Na/Ca exchange is sensitive to membrane potential and cannot extrude Ca during membrane depolarization (Eisner and Lederer, 1985; Mullins, 1981), it follows that the time-averaged efflux of Ca via Na/Ca exchange will be smaller for a fixed interval after an increase in the stimulation frequency. This is because the cell membrane spends more time depolarized. Increasing the beat frequency will both increase the time-averaged influx and reduce the time-averaged efflux, with the result that the intracellular Ca content will increase and contribute to SR content. Contractile tension may also be assumed to increase because there is more SR Ca available for contractile activation. Fabiato (1985) has recently obtained evidence that the slow inward current may contribute to SR loading and that SR loading participates in the force-frequency relationships of the intact mammalian heart.

Depletion and replenishment of the SR after changes in SL Ca transport explain some types of contractile regulation, but are insufficient for others. For example, it is well known that changes in extracellular Ca produce a rapid alteration in the twitch responses in mammalian cardiac muscle. Recently, Kitazawa (1984) has shown that it is possible to alter extracellular Ca rapidly in small bundles (70–120 μM) of guinea pig ventricular muscle. At low rates of electrical stimulation (6 beats/min), the effect of changing extracellular Ca was almost complete within the diastolic phase. More important, an examination of resting leak from the SR (in saponin-skinned fibers) revealed that the SR Ca content could not have changed sufficiently within the diastolic interval to produce the observed effect on tension. Kitazawa (1984) therefore suggests that changes in extracellular Ca alter Ca influx, which in turn alters twitch tension (by affecting Ca-induced Ca release from the SR) rapidly. However, it is also reasonable to expect that changes in extracellular Ca will eventually (by altering transsarcolemmal Ca flux) alter the SR content. In the long run, SR depletion may contribute to the determination of contractile strength. As so elegantly demonstrated by Kitazawa, contractile regulation of heart muscle involves a complex interaction between the short-term and long-term consequences of changing transsarcolemmal Ca transport.

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