Myofilament Ca\(^{2+}\) Sensitivity in Intact Versus Skinned Rat Ventricular Muscle

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Abstract The Ca\(^{2+}\) sensitivity of myofilaments was compared before and after skinning in the same rat trabeculae at a diastolic sarcomere length of 2.2 to 2.3 μm. Trabeculae from rat right ventricle were loaded with fura-2 salt by iontophoretic microinjection, and [Ca\(^{2+}\)] was determined from the epifluorescence at 510 nm when excited at 340 and 380 nm. Steady-state activation was achieved by stimulating the muscle at 10 Hz after 10 to 20 minutes of application of ryanodine (5 μmol/L). The muscles were then skinned with Triton X-100 (1%) for 15 to 25 minutes and subsequently activated with solutions containing [Ca\(^{2+}\)]. The intact force-[Ca\(^{2+}\)] relation was highly cooperative (Hill coefficient, 4.87 ± 0.35, n = 10), with a low [Ca\(^{2+}\)] required for half-maximal activation (K\(_{1/2}\) = 0.62 ± 0.03 μmol/L). After skinning, the Hill coefficient fell to 2.72 and the K\(_{1/2}\) shifted rightward to 2.2 μmol/L in the presence of 1.2 mmol/L free Mg\(^{2+}\). Because of uncertainty regarding the appropriate [Mg\(^{2+}\)], we measured [Mg\(^{2+}\)] at 0.72 ± 0.06 mmol/L (n = 11) with Mg-fura-2 salt. When activating solutions were modified to contain [Mg\(^{2+}\)]=0.5 mmol/L, the force-[Ca\(^{2+}\)] relation was shifted to the left (K\(_{1/2}\) = 0.93 ± 0.1, n = 10) with a Hill coefficient of 3.75 ± 0.37, but the changes were not sufficient to superimpose with the intact force-[Ca\(^{2+}\)] relation (P<0.05 versus intact). These results suggest that, despite the significant effect of Mg\(^{2+}\) on the force-[Ca\(^{2+}\)] relation in skinned muscles, the Ca\(^{2+}\) responsiveness of the myofilaments is still altered by skinning. Possible factors that might underlie the observed discrepancy in the force-[Ca\(^{2+}\)] relation between intact and skinned muscles include decreased Ca\(^{2+}\) binding to troponin (either by loss of natural calcium sensitizers or inadvertent proteolysis), alterations in myosin light chain phosphorylation, and changes in cross-bridge kinetics as a result of skinning. (Circ Res. 1994;74:408-415.)

Key Words • intracellular calcium • intracellular magnesium • excitation-contraction coupling • cardiac muscle

The relation between calcium and force development has been investigated most extensively in “skinned” cardiac muscles, in which the sarcolemmal membrane is removed either chemically or mechanically.1,2 Such preparations have the advantage that [Ca\(^{2+}\)] can be controlled precisely in the activat- ing solution, so that the force-[Ca\(^{2+}\)] relation can be measured directly. Nevertheless, skinning has two major limitations: (1) skinning may result in the loss of important components or regulators of the contractile machinery, and (2) although skinning solutions are designed to mimic the intracellular ionic environment, fundamental uncertainties about ionic strength in vivo and about the correct concentration of some ions (e.g., Mg\(^{2+}\)) make it difficult to set these important regulators of the force-[Ca\(^{2+}\)] relation.3-4 Concerns about skinned fibers were heightened when Yue et al5 demonstrated that the force-[Ca\(^{2+}\)] relation in intact cardiac muscle is much steeper and the [Ca\(^{2+}\)] required for maximal activation an order of magnitude lower than in most skinned fiber studies. Such findings have recently been confirmed by other investigators.6,7

The large difference in the force-[Ca\(^{2+}\)] relation between intact and skinned cardiac preparations has never been confirmed directly in the same muscles, nor is the reason for this apparent discrepancy known. We sought to determine the force-[Ca\(^{2+}\)] relation before and after skinning in the same cardiac muscles. Such an approach has several advantages: (1) maximal Ca\(^{2+}\)-activated force can be compared directly before and after skinning; (2) sarcomere length, which is an important factor affecting force generation,8 can be controlled and kept at the same length before and after skinning; and (3) possible variations from muscle to muscle can be minimized. This study demonstrates that there is indeed a disparity in the force-[Ca\(^{2+}\)] relations obtained before and after skinning. Decreasing [Mg\(^{2+}\)] in the bathing solution to the lowest level that we measured with Mg-fura-2 in this preparation shifted the force-[Ca\(^{2+}\)] relation to the left, but important differences were still detectable after skinning. A preliminary report has appeared.9

Materials and Methods

Preparation

Rats (LBN-F1 brown rats, 200 to 250 g; Harlan, Indianapolis, Ind) were anesthetized with ether and the hearts rapidly excised via a midsternal thoracotomy. Trabeculae from the right ventricle were dissected according to the method described previously.10 The dimensions of the trabeculae were 1.89±0.37 mm long, 0.127±0.045 mm wide, and 0.078±0.021 mm thick (mean±SD, n=22). After dissection, the trabeculae were mounted between a force transducer and a micromanipulator in a perfusion bath. The trabeculae were superfused with Krebs-Henseleit (K-H) buffer equilibrated with 95% O\(_2\)/5% CO\(_2\) gas mixture. The K-H buffer was composed of (in mmol/L): Na\(^+\) 142, K\(^+\) 5, Mg\(^{2+}\) 1.2, Cl\(^-\) 127.4, PO\(_4\)\(^{3-}\) 2, HCO\(_3\)\(^{-}\) 20, CaCl\(_2\) 0.5, and pH 7.35 to 7.4. The perfusion rate was 10 mL/min, and the preparations were stimulated at 0.5 Hz. All the experiments were performed at room temperature (20°C to 22°C).

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Force and Sarcomere Measurements

Force was measured with a silicon strain gauge (AEM 801, SensoNor, Horten, Norway). A basket specially designed to hold the carnal end of the trabecula was glued to the strain gauge. The valve end was hooked onto a stainless steel needle attached to the micromanipulator, which was set to the desired muscle length. Sarcomere length was measured as described by ter Keurs et al. A laser beam was projected onto the muscle. The first order of the diffracted light was collected by a video camera, and the image of the diffraction was displayed on a video monitor. After careful calibration with standard grating, sarcomere length could be measured from the video image. Diastolic sarcomere length was set to 2.2 to 2.3 μm and monitored throughout the experiments.

Measurement of Intracellular [Ca^{2+}] With Fura-2

Intracellular [Ca^{2+}] was measured by the method recently developed by Backx and ter Keurs.11 Fura-2 potassium salt was microinjected iontophoretically into one cell and allowed to spread throughout the whole muscle (via gap junctions). The tip of the microelectrode (~0.2 μm in diameter) was filled with fura-2 salt (1 mmol/L), and the microelectrode was back-filled with 150 mmol/L KCl. Electrodes had resistances of 200 to 270 MΩ when placed in K-H buffer. After a successful impalement into a superficial cell in the unstimulated muscle, a hyperpolarizing current of 5 to 8 nA was passed continuously for 20 to 30 minutes. After the injection, fura-2 was initially localized around the site of injection. The preparation was stimulated at 0.5 to 1 Hz for 40 to 60 minutes, after which the preparation was uniformly loaded with fura-2.

Intracellular [Ca^{2+}] was determined by measuring the epifluorescence of fura-2 in the cells. Excitation UV light filtered by bandpass filters (bandwidth, 10 nm) of Oriel, Stratford, Conn) at 380, 360, and 340 nm was projected onto the muscle via a 10× objective (10× Fluor, Nikon, Tokyo, Japan) using a dichroic mirror (400 DPLC, Nikon). The UV light illuminates an area ~1.2 mm in diameter in the focal plane that contains the muscle. The fluorescent light was collected at 510 nm by a photomultiplier tube (R1527, Hamamatsu, Bridgeport, NJ). The output of the photomultiplier tube was filtered at 100 Hz, collected by an analog-to-digital converter, and stored in the computer for later analysis.

Intracellular [Ca^{2+}] was given by the following equation (after subtraction of the autofluorescence of the muscle):

\[
[Ca^{2+}] = K_d (R - R_{\text{min}})/(R_{\text{max}} - R)
\]

where R is the observed ratio of fluorescence (340/380), \(K_d\) is the apparent dissociation constant, \(R_{\text{min}}\) is the ratio of 340/380 nm at saturating \([Ca^{2+}]\), and \(R_{\text{max}}\) is the ratio of 340/380 nm at zero \([Ca^{2+}]\).12 The values for \(K_d\), \(R_{\text{min}}\), and \(R_{\text{max}}\) were determined by in vivo calibrations (see below).

In Vivo Calibration of Fura-2

In vivo calibrations were carried out by a previously described method.11,12 After loading fura-2, the muscle was poisoned with a solution containing (in mmol/L): KCl 140, HEPES 25, MgCl2 0.75, NaCN 2, and iodoacetic acid (IAA) 0.5, at pH 7.4 and room temperature. About 10 minutes after full rigor development, solutions containing (in mmol/L): K2EGTA 10, KCl 100, HEPES 25, MgCl2 0.75, NaCN 2, and IAA 0.5 and 50 μmol/L ionomycin (Calbiochem, San Diego, Calif) with varied \([Ca^{2+}]\) were applied to the bath. \([Ca^{2+}]\) was varied by mixing K2EGTA and CaEGTA proportionally (see below). Fig 1 shows the in vivo calibration curve. The \(R_{\text{max}}\) and \(R_{\text{min}}\) were 9.8 and 0.53, respectively. The apparent \(K_d\) was 2.52 μmol/L. These values are similar to those obtained in previous calibrations both in vitro and in vivo.11

Measurement of Intracellular [Mg^{2+}] Using Mg-Fura-2 Potassium Salt (Furaprat)

The methodology for measuring intracellular \([Mg^{2+}]\) was the same as for intracellular \([Ca^{2+}]\) except that fura-2 salt was replaced by furaprat (Mg-fura-2) salt (Molecular Probes, Eugene, Ore). Calibration of furaprat salt was done in vitro using solutions containing 140 mmol/L KCl, 25 mmol/L HEPES, 200 μmol/L BAPTA, and MgCl2 in the presence of 0.5 μmol/L furaprat salt. Since furaprat also binds to \(Ca^{2+}\) (true \(K_d\) for \(Ca^{2+}\), 10 to 50 μmol/L), we minimized the contribution of \(Ca^{2+}\) to the measured values by determining \([Mg^{2+}]\) from the 340/380 ratio at end diastole, when \([Ca^{2+}]\), is <200 mmol/L and thus binds negligibly to furaprat.

Skinning of Trabeculae

After determination of the intact force-[\(Ca^{2+}\)] relation, the trabeculae were skinned in the same bath with 1% Triton X-100 for 15 to 25 minutes in relaxing solution containing (in mmol/L): KCl 100, HEPES 25, MgCl2 0.5, Na2ATP 5, and MgCl2 5.15. The purity of EGTA was determined with a pH-metric technique as described by Miller and Smith13 before use. Leupeptin (0.5 mmol/L), a protease inhibitor, was also added to the solutions to keep the skinned muscles from deteriorating.

The pH was adjusted to 7.2 with KOH (~15 mmol/L). The progress of skinning was monitored by direct visualization and was stopped when the preparation had lost all its pink color. The muscle was then bathed in relaxing solution without Triton X-100. In several experiments, the skinning was further verified by the absence of contraction upon addition of 20 mmol/L caffeine to the bath. Varied \([Ca^{2+}]\) was achieved by mixing the relaxing solution and activating solution (in mmol/L: CaEGTA 10, KCl 100, HEPES 25, Na2CrP 15, Na2ATP 5, MgCl2 4.75, and leupeptin 0.5, at pH 7.2). \([Ca^{2+}]\) was calculated by a computer program based on the stability constants and the enthalpy values for the various reactions from Martell and Smith,14 except values for Mg-ATP and Ca-ATP reactions from Pettit and Siddiqui.15 The muscles were activated with varied \([Ca^{2+}]\) solutions, while sarcomere length was kept the same as before skinning.

Statistics

Student's t test was used for the statistical analysis of the data.16 A value of \(P<.05\) was considered statistically significant.
Results

Steady-State Force-[Ca\textsuperscript{2+}] Relation in Intact Trabeculae

The steady-state force-[Ca\textsuperscript{2+}] relation was first determined in intact trabeculae. Fig 2 shows twitch force, tetanized force, and the underlying changes in intracellular [Ca\textsuperscript{2+}] in one trabecula loaded with fura-2 salt. Sarcomere length was set at 2.2 μm. Tetanization of the trabecula was achieved by addition of ryanodine (5 μmol/L) and increasing the stimulus rate to 10 Hz. Different levels of tetanized force were obtained by increasing [Ca\textsuperscript{2+}] in the perfusion solution (up to 30 mmol/L). Upon stimulation at 10 Hz, tetanized force developed gradually and reached a plateau in about 1 to 2 seconds, whereas intracellular [Ca\textsuperscript{2+}] rose rapidly in the first few milliseconds and then more slowly to a stable level. As expected, at maximal activation, force remained unchanged despite increases in [Ca\textsuperscript{2+}].

Fig 3 shows the steady-state force-[Ca\textsuperscript{2+}] relation of this muscle. Maximal activation was achieved at 1 μmol/L [Ca\textsuperscript{2+}]. The [Ca\textsuperscript{2+}], required for half-maximal activation (K_{0.5}) was 0.6 μmol/L, and the Hill coefficient was 5. In this muscle, maximal [Ca\textsuperscript{2+}], achieved by the protocol was 1.4 μmol/L. Higher [Ca\textsuperscript{2+}], (3 to 4 μmol/L) was obtained in two other muscles by addition of 1 μmol/L Bay K 8644, but this was not done routinely because the drug was very difficult to wash out from the bath.

Force-[Ca\textsuperscript{2+}] Relation Before and After Skinning

The type of force-[Ca\textsuperscript{2+}] relation shown in Fig 3 is typical of many muscles\textsuperscript{17} but clearly differs from most results from skinned muscles.\textsuperscript{18} Although such differences have been appreciated, they have not been demonstrated or investigated by paired analysis. Comparison with published skinned muscle data is further complicated by differences in the force-[Ca\textsuperscript{2+}] relations among studies because of different experimental conditions, even in the same species (eg, in rat cardiac muscle the K_{0.5} varied from 2 to 6 μmol/L and Hill coefficients from 1.9 to 3).\textsuperscript{19} We therefore performed experiments in which we determined the force-[Ca\textsuperscript{2+}] relation before and after skinning in the same muscles. As an internal consistency check, we first determined whether or not the maximal Ca\textsuperscript{2+}-activated force was the same before and after skinning. It is known that maximal activation and Ca\textsuperscript{2+} sensitivity of the myofilaments are affected by a number of factors (eg, sarcomere length, pH, phosphate, and ionic strength).\textsuperscript{4,8,19-21} Since maximal activation defines the upper range of the force-[Ca\textsuperscript{2+}] relation, it is imperative to keep these factors as close as possible to the intact situation to minimize their effects. Under basal conditions in mammalian hearts, intracellular pH and intracellular inorganic phosphate concentration have been measured to be 7.1 to 7.2 and <0.2 mmol/L, respectively.\textsuperscript{22} These two factors are controlled and set appropriately in our solutions. Sarcomere length was kept the same before and after skinning. Unfortunately, the value of ionic strength in the cytosol of intact muscle is unknown. However, since ionic strength affects the maximal Ca\textsuperscript{2+}-activated force,\textsuperscript{4} differences in the maximal force should reflect differences in ionic strength between intact muscle and the solutions. Fig 4 shows tetanized force and [Ca\textsuperscript{2+}] during maximal activation before skinning (A) and maximal activation (with 25 μmol/L Ca\textsuperscript{2+}) after skinning (B) from the same muscle. The same maximal level of activation was achieved in both cases. In five trabeculae in which the force-[Ca\textsuperscript{2+}] relation was determined before and after skinning, the maximal force before skinning was 93±12 mN/mm\textsuperscript{2} before and 90±12 mN/mm\textsuperscript{2} after skinning (mean±SEM, P=.9). The excellent agreement provides considerable reassurance that the ionic conditions in our activating solutions were not very different from those in intact muscles.

Two trabeculae in which the intact force-[Ca\textsuperscript{2+}] relation was determined were then skinned with 1% Triton X-100, and the force-[Ca\textsuperscript{2+}] relation was defined

\[
\text{Force} = F_{0.5}(\text{Ca}^{2+}) \cdot \frac{K_{0.5} \cdot [\text{Ca}^{2+}]}{[\text{Ca}^{2+}]^2 + K_{0.5}}
\]

where F_{0.5} is the maximal force, K_{0.5} is half the maximal force, and n is the Hill coefficient. In this trabecula, F_{0.5}=81 mN/mm\textsuperscript{2}, K_{0.5}=0.6 μmol/L, and n=4.7.
Afterward, the muscle was skinned with 1% Triton X-100 in relaxing solution (see "Materials and Methods"). Maximal force was achieved by bathing the skinned trabecula in solution containing 25 μmol/L [Ca^{2+}]. Note the virtual identity in the magnitude of force at maximal activation before and after sking. Preparation 160693.

in the presence of 1.2 mmol/L free Mg^{2+} and pH 7.0 (Fig 5). These [Mg^{2+}] and pH values were chosen for consistency with most previous skinned fiber studies to determine whether the large differences in the steepness and position of the force-[Ca^{2+}] relations between independent studies either in intact or in skinned muscles could be demonstrated in the same muscle. As shown in Fig 5, the differences in the steepness and position of the force-[Ca^{2+}] relation before and after skinning are readily apparent. Before skinning, K_{1/2} was 0.72 μmol/L with a Hill coefficient of 6.2, whereas after skinning, K_{1/2} increased to 2.2 μmol/L while the Hill coefficient fell to 2.7.

**Effect of Mg^{2+} Level on Force-[Ca^{2+}] Relation in Skinned Cardiac Muscle**

The above results obtained in the same muscles show directly that there are disparities in the force-[Ca^{2+}] relations in intact versus skinned cardiac muscles. The question to ask, then, is what is the origin of these changes? In this study, sarcomere length, which affects the force-[Ca^{2+}] relation, was measured and kept the same before and after skinning. Furthermore, the ionic conditions were chosen such that they had no effect on maximal Ca^{2+}-activated force. This would at least minimize the effects of factors such as pH, inorganic phosphate, and ionic strength, which are known to be possible sources of error in matching the solutions used in skinned fibers to the intact cytoplasm. Fig 5 suggests that additional factor(s) may play a role in the differences in the force-[Ca^{2+}] relations before and after skinning. Among the known factors that affect the force-[Ca^{2+}] relation, Mg^{2+} merits further consideration for two obvious reasons: (1) Despite its significant impact on the force-[Ca^{2+}] relation (elevation of [Mg^{2+}] in the millimolar range increases K_{1/2} and decreases the Hill coefficient), Mg^{2+} has no effect on the maximal Ca^{2+}-activated force; and (2) the intracellular Mg^{2+} concentration is not well established. In light of the effect of Mg^{2+} on the force-[Ca^{2+}] relation and the uncertainties about its intracellular level, we performed additional experiments to see whether Mg^{2+} is the causative factor.

We first measured intracellular free Mg^{2+} concentration using furaptra salt in the same way as we measured intracellular [Ca^{2+}] in our trabeculae. Fig 6A shows the in vitro calibration of furaptra. R_{max} (340/380) was 2.02, R_{min} was 0.37, and the apparent K_{d} was 16.33 mmol/L as determined by the best fit of the least-squares function. The calculated true K_{d} (the apparent K_{d} divided by the ratio of fluorescence of the Mg^{2+}-free to Mg^{2+}-bound forms of the dye when excited at 380 nm) was 2.28 mmol/L. We also attempted in vivo calibration using a similar approach as with fura-2 salt, but we did not convincingly achieve equilibration of Mg^{2+} across the membrane with ionomycin. We therefore used the K_{d}, R_{max}, and R_{min} from our in vitro calibrations to estimate intracellular Mg^{2+} concentration in our trabeculae. The validity of this approach is supported by the study of Westerblad and Allen, in which superimposable in vitro and in vivo calibration curves were obtained when the in vivo calibration was performed by pressure-injecting MgCl_{2} into skeletal muscle fibers. In our intact trabeculae, intracellular [Mg^{2+}] was found to be 0.72±0.06 mmol/L (n=11); range, 0.5 to 1.1 mmol/L, which is consistent with previous studies in intact heart. Intracellular [Mg^{2+}] was not affected by contraction or by increasing [Ca^{2+}] in the perfusion solution (Fig 6B). Clearly, intracellular free [Mg^{2+}] is lower than the values used previously in most skinned muscle studies (>1 mmol/L).

We then performed experiments to evaluate the effects of Mg^{2+} on the force-[Ca^{2+}] relation. We chose the lowest [Mg^{2+}] we measured in our muscles (0.5 mmol/L instead of the average value of 0.72 mmol/L) and a slightly higher pH (7.2 instead of 7.0) in an attempt to achieve the greatest plausible leftward shift of the force-[Ca^{2+}] relation, in light of the pronounced...
The solution free Mg normalized skinning $K_{112}$ was of convenience for many experiments (all genera). Figure 6 shows the results from pooled data before and after skinning, with low [Mg$^{2+}$] in the activating solution. The paired data from Figure 7 are included, as well as results from several unpaired trabeculae that were studied only in the intact or skinned conditions. In intact muscles (n=10), $K_{112}$ was 0.62±0.03 (mean±SEM), the Hill coefficient was 4.87±0.35, and maximal activation was achieved at [Ca$^{2+}$] of <$1 \mu$mol/L. In skinned muscles at 0.5 mmol/L Mg$^{2+}$ (n=10), the $K_{112}$ was 0.93±0.1 (P<0.01 versus intact), the Hill coefficient was 3.75±0.37 (P<0.04 versus intact), and [Ca$^{2+}$] required for maximal activation was >4 mmol/L. The data indicate that the sensitivity of the myofilaments to Ca$^{2+}$ is altered by skimming even when care is taken to keep [Mg$^{2+}$] as low (and pH as high) as is plausible given contemporary measurements of these variables in intact preparations.

**Discussion**

**Force-[Ca$^{2+}$] Relation in Cardiac Muscle**

**Force-[Ca$^{2+}$] Relation of Intact Trabeculae**

Skinned fibers have provided the vast majority of our information regarding the force-[Ca$^{2+}$] relation of cardiac muscle. Recent studies, however, have used intact muscles.$^5,7,17$ The agreement in the force-[Ca$^{2+}$] relations of intact muscles in the present study and in previous studies is striking, especially when one considers the complementary strengths and limitations of the various methods. For example, Figure 8 (closed circles) is superimposable with the results of Yue et al.$^8$ and Okazaki et al.$^8$ despite differences in preparations (ferret papillary muscles versus rat trabeculae) and in the techniques for intracellular [Ca$^{2+}$] measurement (aequorin versus fura-2). The $K_{112}$ and Hill coefficient in the

Fig 8. Graph showing pooled data of force-[Ca$^{2+}$] relation in intact muscles (a) and after skinning (c). In intact muscles (n=10), $K_{112}$ was 0.62±0.03, and the Hill coefficient was 4.87±0.35. In skinned trabeculae at 0.5 mmol/L free Mg$^{2+}$ (n=10), $K_{112}$ was 0.93±0.1 (P<0.01 vs intact), and the Hill coefficient was 3.75±0.37 (P<0.04 vs intact). Preparations 01192, 251192, 10093, 18093, 1706W, 051092, 271092, 280193, 290193, 120192, 12093, 13093, 14093, 15093, 200493a, 220493b, 230493.
present study are 0.62 \(\mu\)mol/L and 4.87, respectively, which are virtually identical with previous studies.\(^5\)\(^7\) All these studies also showed that maximal Ca\(^{2+}\) activated force was achieved at [Ca\(^{2+}\)] below 1 \(\mu\)mol/L. Thus, the salient features of the force-[Ca\(^{2+}\)] relation in intact muscle are readily reproducible.

Although intact muscles have some clear advantages, they obviously afford less rigorous control of the cytosolic constituents than do skinned muscles. It is possible that what one actually measures in intact muscle are points on a family of Ca\(^{2+}\)-force relations resulting from changing metabolic conditions during tetany. The magnitude of such changes is likely to be very small: Kusuoka et al\(^5\) have determined that the changes in inorganic phosphate and pH during 4.3 seconds of maximal tetanic activation in ferret hearts are modest, producing a <10% decline in maximal force ("fatigue").

The tetany in the present study did not exhibit any fatigue, arguing against significant metabolite accumulation. Although changes in myofilament sensitivity have been reported during very long tetany (20 to 40 seconds) in single cells, no such effects are evident with tetany of the short duration used here.\(^6\) Finally, the Ca\(^{2+}\)-force relations measured as described here superimpose with those determined from the slow relaxation of single-twitch contractions evoked in the presence of cyclopiazonic acid, an inhibitor of Ca\(^{2+}\) uptake by the sarcoplasmic reticulum.\(^7\) This correspondence is reassuring because the properties of the myofilaments are not likely to change much during the course of a single twitch contraction.

**Force-[Ca\(^{2+}\)] Relation of Skinned Trabeculae**

The force-[Ca\(^{2+}\)] relation from skinned trabeculae is less steep and shifted to the right compared with that of intact muscles (Figs 7 and 8). The differences may arise from either of two general factors: failure of the solutions used in skinning studies to duplicate the cytoplasmic environment, or fundamental changes in the myofilaments as a result of skinning. Indeed, there are noticeable variations in the cardiac force-[Ca\(^{2+}\)] relation among different species and within the same species under different experimental conditions.\(^18\) Whereas most studies have shown a K\(_{1/2}\) of 2 to 6 \(\mu\)mol/L with a Hill coefficient of 2 to 3, extreme deviations in either direction have also been reported.\(^19\)\(^20\) Our data from skinned trabeculae at 1.2 mmol/L Mg\(^{2+}\) (Fig 4) are comparable with the results from most previous studies under similar conditions.\(^18\) Likewise, the data in Fig 8 are comparable with Fabiato and Fabiato's results\(^3\) using low [Mg\(^{2+}\)] in the activating solutions in rat ventricular cells. The effect of pH is less clear because at both pHs the maximal Ca\(^{2+}\)-activated force was equal. We also believe that (although not proven) we have set up an optimal leftward-shift condition for the skinned trabeculae because we used a lower [Mg\(^{2+}\)] and higher pH compared with most previous studies in cardiac muscle.

**Factors Responsible for the Disparity in the Force-[Ca\(^{2+}\)] Relation Between Intact and Skinned Muscles**

**Loss of Natural ‘Ca\(^{2+}\) Sensitizers’ by Skinning**

Skinning removes the physical barrier (sarcolemma) between the cytoplasm and the external solution, resulting in the loss of substances from the cytoplasm. These include carnosine-like compounds and taurine, which are known as natural "Ca\(^{2+}\) sensitizers."\(^29\)\(^30\) The carnosine-like compounds (or histidyl derivatives) have been found to be present in the millimolar concentration range in cytoplasm, high enough to cause an increase in Ca\(^{2+}\) sensitivity despite the fact that the effect is mild.\(^29\) It is worthwhile to note that these compounds increased maximal Ca\(^{2+}\)-activated force only modestly (from 1.5% to 15%) while shifting the force-[Ca\(^{2+}\)] relation to the left (by \(\approx\)500 mmol/L) at submaximal activation. Taurine is another natural Ca\(^{2+}\) sensitizer present in the cell, which was also shown to increase Ca\(^{2+}\) sensitivity without affecting maximal force.\(^30\) However, these two compounds did not increase the Hill coefficient.

The mechanism by which these compounds increase Ca\(^{2+}\) sensitivity is not clear. Mg\(^{2+}\) and low pH reduce Ca\(^{2+}\) sensitivity of the myofilaments by decreasing the affinity of troponin C to Ca\(^{2+}\).\(^31\)\(^32\) While sulmazole, a chemical Ca\(^{2+}\) sensitizer that also has a histidyl ring in its molecular structure, increases Ca\(^{2+}\) sensitivity by increasing Ca\(^{2+}\) binding to troponin C.\(^33\) By analogy, it seems likely that carnosine and related compounds increase Ca\(^{2+}\) sensitivity by increasing the affinity of troponin C to Ca\(^{2+}\). Nevertheless, these compounds cannot account fully for the discrepancies we have observed between skinned and intact muscles, given that they do not change the Hill coefficient. Further investigation will be required to evaluate the importance of endogenous Ca\(^{2+}\) sensitizers, especially by careful comparison of intact and skinned muscles.

**Inadvertent Proteolysis in Skinned Muscles Exposed to High [Ca\(^{2+}\)]**

It is known that cardiac cells contain at least two forms of Ca\(^{2+}\)-activated neutral protease or calpain.\(^34\) Experimentally, calpain II has been shown to digest various components of the myofilaments (including troponin T and I, myosin, and C protein; see Mellgren and Murachi\(^34\) for references) when activated at millimolar [Ca\(^{2+}\)]. We have observed that skinned trabeculae exposed to calpain I at 10 \(\mu\)mol/L [Ca\(^{2+}\)] exhibited a marked decrease in Ca\(^{2+}\) sensitivity.\(^35\) However, maximal force was also decreased, unlike the present findings. The extent to which proteolysis contributes to the changes in Ca\(^{2+}\) sensitivity after skinnning is less clear in the present experiments, especially given that leupeptin, an effective protease inhibitor, was present in our skinnning solutions. Furthermore, the specific substrate of calpain I on the myofilaments is not known.

**Phosphorylation of Myosin Light Chain**

In skinned cardiac fibers, incubation with myosin light chain (MLC) kinase shifts the force-[Ca\(^{2+}\)] relation to the left without an effect on the maximal Ca\(^{2+}\)-activated force caused by the Ca\(^{2+}\)-activated calmodulin-dependent phosphorylation of MLC.\(^36\)\(^37\) On the other hand, dephosphorylation of MLC with light chain phosphatase decreases the Ca\(^{2+}\) sensitivity without affecting maximal force. This mechanism was shown to be independent of the \(\alpha\) - and \(\beta\)-adrenergic systems.\(^38\)\(^39\) Although there is no direct evidence that such a mechanism is responsible for the high Ca\(^{2+}\) sensitivity in intact muscle, it is possible that this mode of modulation of
Ca\(^{2+}\) sensitivity is impaired after skinnning because of the loss of calmodulin and (possibly) MLC kinase.

**Cross-Bridge Kinetics and Lattice Spacing of Myofibrils**

Brenner\(^4\) has proposed another troponin C-independent mode of Ca\(^{2+}\) sensitivity regulation: Ca\(^{2+}\) modulation of cross-bridge turnover kinetics (or rate modulation). In this mode of regulation, the rate of transition of cross-bridges to force-generating states depends on [Ca\(^{2+}\)] and dominates the shape of the force-[Ca\(^{2+}\)] relation, whereas maximal Ca\(^{2+}\)-activated force remains unchanged. Sweeney and Stull\(^4\) showed that MLC phosphorylation increased the rate of transition of cross-bridges to force-generating states. If the phosphorylation of MLC is lost or diminished by skinning, the kinetics of cross-bridge turnover are expected to change.

Removal of the sarcolemma resulted in swelling of the fibers from skeletal muscle,\(^2,4,4\) thus causing increases in the lattice spacing of the myofilaments as revealed by x-ray diffraction.\(^4\) It is possible that cross-bridge properties, especially cross-bridge kinetics, would change, resulting in a decrease in Ca\(^{2+}\) sensitivity. Hysteresis in the force-[Ca\(^{2+}\)] relation, a phenomenon observed in skinned muscle fibers\(^45\) that is eliminated by lattice shrinkage,\(^46\) could in principle contribute to the differences between intact and skinned muscles. However, this mechanism is not likely to be prominent under our experimental conditions: first, the experiments were done at sarcomere lengths of 2.2 to 2.3 \(\mu m\), at which hysteresis has been shown to be absent;\(^2\) second, lattice shrinkage,\(^46\) could in principle contribute to the differences between intact and skinned muscles.

Moreover, this mechanism is not likely to be prominent under our experimental conditions: first, the experiments were done at sarcomere lengths of 2.2 to 2.3 \(\mu m\), at which hysteresis has been shown to be absent;\(^2\) second, lattice shrinkage,\(^46\) could in principle contribute to the differences between intact and skinned muscles.

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Thus, this study has demonstrated that (1) Ca\(^{2+}\) sensitivity of the myofilaments is reduced by skinning, (2) Mg\(^{2+}\) is an important factor in the regulation of Ca\(^{2+}\) sensitivity in skinned muscle fibers, and (3) other regulatory mechanisms lost or altered by skinning may play a role in the higher Ca\(^{2+}\) sensitivity seen in intact muscles. After correction for [Mg\(^{2+}\)], the major discrepancy between intact and skinned muscles lies in the degree of cooperativity, which is blunted after skinning. Since the "foot" of the force-[Ca\(^{2+}\)] relation is not shifted, the decrease in cooperativity makes for a less steep relation with a higher \(K_{1/2}\) in the skinned muscle. Further studies are needed to define the origin of these changes.

Our findings indicate that caution is merited in extrapolating results from skinned muscles directly to intact preparations. The potential pitfalls are likely to be greatest in attempts to reconcile measurements of [Ca\(^{2+}\)], with physiological levels of contractile activation: Ca\(^{2+}\) transients have always seemed to be too small to produce appreciable force when viewed in light of the bulk of the steady-state force-[Ca\(^{2+}\)] relations from skinned muscles. No such discrepancy occurs when Ca\(^{2+}\) transients are compared with the intact steady-state force-[Ca\(^{2+}\)] relations reported here and elsewhere.\(^5,7\) Nevertheless, the utility of skinned muscle studies remains unquestionable: thus far, directional changes induced by interventions such as ions and drugs have all proved to be accurately predicted by skinned muscle studies. Furthermore, much remains to be learned about the mechanism of cross-bridge cycling, and it is difficult to imagine that control of experimental conditions adequate to address this issue can be achieved without skinning. Although we caution against a reckless rejection of skinned muscle data, our findings highlight the importance of close correlation of such data with results from intact preparations.

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