Length-dependent activation in intact ferret hearts: study of steady-state Ca^{2+}-stress-strain interrelations

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Stennett, Richard, Kazuhide Ogino, James P. Morgan, and Daniel Burkhoff. Length-dependent activation in intact ferret hearts: study of steady-state Ca^{2+}-stress-strain interrelations. *Am. J. Physiol.* 270 (*Heart Circ. Physiol.* 39): H1940–H1950, 1996.—We examined the steady-state stress-strain relationships and the steady-state stress-intracellular calcium concentration ($[Ca^{2+}]_i$) relationship in intact ferret hearts and compared these to previously published analogous relationships in skinned and intact muscle. Langendorff-perfused ferret hearts were treated with ryanodine and tetanized by rapid stimulation to create steady-state conditions. $[Ca^{2+}]_i$ was measured concurrently by macroinjected aequorin. Over a range of volumes corresponding to strains between 1.0 and 0.75, steady-state stress decreased by 33% when saturating levels of perfusate calcium were used, indicating the degree to which physical factors contribute to the Frank-Starling relationship. The steady-state stress-$[Ca^{2+}]_i$ relationship was sigmoidal with a mean Hill coefficient (n_H) of 4.91 ± 0.29 at a strain of 1.0, and the $[Ca^{2+}]_i$ required for half-maximal activation ($K_{½}$) was 0.41 ± 0.03 μM. $K_{½}$ increased and n_H decreased with decreasing strains. These results are similar to those observed in intact muscle but differ quantitatively from results obtained in isolated, skinned preparations. Based on these results, we suggest that whole heart function can be related to average sarcomere function without the need for complex models of ventricular structure.

ventricular mechanics; excitation-contraction coupling; calcium; aequorin; ferret; ryanodine; sarcomere

STUDIES OF ISOLATED INTACT and skinned cardiac muscle have yielded a great deal of information about how muscle length influences myofilament properties and, consequently, modifies force generation (1, 17, 22, 23, 27). Despite this wealth of knowledge, and despite the fact that this aspect of cardiac muscle physiology underlies an analogous property at the organ level, the so-called Frank-Starling mechanism, description, and evaluation of ventricular properties are largely limited to phenomenological descriptions based on pressure-volume analysis, such as the time-varying elastance and end-systolic pressure-volume relationship (32). With growing appreciation for the limitations of these phenomenological approaches (6, 7, 13, 14, 24, 26, 34, 35), however, it has been proposed that basic concepts of cardiac muscle contraction can be used to explain whole heart behavior (5, 15).

Two fundamental relationships that characterize myofilament properties under a steady state of calcium activation are *1*) the steady-state force-calcium relationship and *2*) the steady-state force-length relationship. Several important concepts have been gleaned from these relations. First, cross-bridge coupling (force generation) is a cooperative process with regard to calcium binding. Second, muscle length modifies myofilament calcium responsiveness. Third, even with maximal calcium activation, changes in muscle length over a broad range of sarcomere lengths leads to changes in force. Although the mechanisms of these phenomena are still unclear, these observations and concepts form the foundation for a more mechanistic description of dynamic ventricular properties.

However, because of divergent experimental conditions, complex ventricular ultrastructure, potential heterogeneity of contractile properties, and dispersion of sarcomere lengths, the question is raised as to whether concepts derived from isolated muscle could be used to describe whole heart function without the use of detailed mathematical models that account for these factors. Availability of techniques to measure intracellular calcium concentration ($[Ca^{2+}]_i$) from small regions of intact hearts (28) and the ability to tetanize the heart (29) now allow for exploration of these important phenomena in intact perfused ventricles.

The purpose of this study was to explore, in the intact heart, the interrelationships among $[Ca^{2+}]_i$, left ventricular (LV) volume (strain), and LV pressure (LVP; stress) under equilibrium (i.e., steady-state) conditions. Specifically, two interrelated relationships were assessed: *1*) the relationship between steady-state pressure and volume at different perfusate calcium concentrations ($[Ca^{2+}]_o$) and *2*) the relationship between steady-state pressure and $[Ca^{2+}]_i$ at different ventricular volumes. Results were compared with analogous results obtained previously in isolated skinned and intact trabecular tissue.

METHODS

Surgical Preparation

In each experiment, an 8- to 14-wk-old ferret (1.2–1.4 kg body wt) was heparinized (1,000 U ip) and then anesthetized with ketamine (100 mg/kg ip) and xylazine (1 mg ip). Bilateral sternotomy was performed, and the heart was rapidly excised and immediately submerged in oxygenated, warmed, modified Tyrode solution (34°C; composition provided below). The severed end of the aorta was fed over a 16-gauge needle that was connected to a modified Langendorff perfusion system, and perfusate flow was adjusted to provide a perfusion pressure of ~70–80 mmHg. The left atrium was opened. A latex balloon attached to the end of a piece of stiff polyethylene tubing, with fenestrations on the distal 5 mm of its tip, was inserted into the LV and held in place by a 2–0 silk pursestring suture placed around the mitral annulus. The balloon and tubing were filled with water and connected to a

Statham pressure transducer for measurement of LVP. Balloon volume could then be varied, using a calibrated 2-ml syringe. Note that the volume of the balloon wall plus the tip of the tubing within the balloon was measured by water displacement after withdrawing all fluid from within the balloon (typical range: 0.8–1.0 ml). This value was added to the volume infused inside the balloon to obtain total LV volume (LVV). Two pacing electrodes were positioned in the right ventricular outflow tract and the right atrium.

The perfusion system consisted of a warmed storage vat for perfusate solutions, an adjustable-speed rotary pump (Masterflex), and a Pyrex condenser. The vat and condenser were warmed by a constant-temperature circulator (VWR) set to heat the solutions to 34°C. Perfusate was composed of (in mM) 15 glucose, 140 NaCl, 5 KCl, 0.9 $MgCl_2$, 2.0 $CaCl_2$, and 6 *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES). The pH was adjusted to 7.40, and the solution was equilibrated with 100% O_2. Perfusate was not recirculated. After attachment of the heart to the modified Langendorff perfusion system, ventricular volume was adjusted to provide an end-diastolic pressure (EDP) of ~10 mmHg, and the hearts were allowed to stabilize for at least 20 min. The end of the stabilization period was defined as the time when peak LVP and coronary perfusion pressure attained a stable level.

Ryanodine Perfusion and Tetanization of Whole Hearts

After stabilization of the isolated heart in modified Tyrode solution containing a 2 mM [Ca^{2+}], ryanodine (3 μM) was added to the perfusate and maintained for 20 to 30 min until the developed pressure, which decreased significantly with ryanodine infusion, remained unchanged for several minutes (29). To elicit tetanizations on demand, rectangular pulses of 50-ms duration and 1.5× voltage threshold at 10 Hz were used as originally described by Marban et al. (29). Tetanic stimulation was maintained for up to 5 s, and maximum developed pressure (defined as steady-state pressure) was achieved after ~1.5 s. Rest periods of at least 2 min, during which time the heart contracted spontaneously, were imposed between successive tetani.

Aequorin Macroinjection, Signal Recording, and Calibration of Aequorin Light Signals

Techniques for measuring calcium transients from the epicardial surface of crystalloid-perfused hearts were similar to those described previously (3, 28). An aequorin solution (composition in mM: 154 NaCl, 5.4 KCl, 1 $MgCl_2$, 12 HEPES, 11 glucose, and 0.1 EDTA and aequorin 1 mg/ml; adjusted to pH 7.40) was prepared. Near the end of the ryanodine infusion (when the heart became quiescent), 3–5 μl of this solution were injected just under the epimysium in the inferoapical region using a low-resistance glass micropipette with an inner diameter of ~30 μm. This was accomplished by performing six separate injections (0.5–1.0 μl each) into an ~3-mm^2 area. It has been shown that a small fraction of this aequorin is "loaded" into myocytes by an unknown mechanism with minimal damage to the cells near the injection site (as evidenced by light microscopy; 28). In the original reports describing this technique, the hearts were exposed to a low calcium concentration solution during the aequorin loading procedure. This has been found to be unnecessary and was therefore not used in the present study.

To record the aequorin luminescence, the heart and a portion of the perfusion apparatus were placed inside a light-tight box that is identical in design to that originally used for aequorin experiments on papillary muscles (4) and described in detail previously (28; Gary Harrar Associates, Rochester, MN). The heart was positioned within a specially designed glass organ bath with a concavity at its base; the inferoapical region of the heart (the aequorin injection site) was placed in contact with this base so that the aequorin luminescence was emitted through the bottom of the bath. The bottom of the organ bath was, in turn, positioned at the focal point of an ellipsoidal light collector that directed the light to the surface of a photomultiplier tube (9235QA, Thorn EMI, Fairfield, NJ). The photomultiplier was energized by a power supply (PM28R, Thorn EMI) with the voltage adjusted to provide an optimal signal-to-noise ratio (900 V). The aequorin light signal was recorded as anodal current with zero set as the mean dark current. Filtering was performed online using an analog filter with a corner frequency of 100 Hz.

The method of calibrating the light signal into an absolute $[Ca^{2+}]_i$ was the same as that used in papillary muscles and has been described previously (28). At the end of the experiment, the heart was perfused with a 50 mM calcium-5% Triton X-100 solution, which lysed the cells and exposed the remaining aequorin to high amounts of calcium (3). Luminescence signals to be converted to calcium signals, L, were normalized by the total light emission, L_{max}, which was estimated as the integral of the aequorin signal collected during the lysis procedure multiplied by the rate constant for aequorin consumption (2.11/s; 28). The instantaneous L/L_{max} was then converted to time-varying $[Ca^{2+}]_i$ according to the equation

$$L/L_{max} = (1 + K_r[Ca^{2+}]_i)/(1 + K_{tr} + K_r[Ca^{2+}]_i)^3 \quad (1)$$

where the rate constants in the relationship between light and calcium concentration K_r and K_{tr} are 4.5×10^6/mol and 130, respectively. Values of K_r and K_{tr} were determined previously (28).

Experimental Protocols

All experiments were performed at 34°C, and all hearts were perfused with ryanodine (3 μM) solution before starting the protocol. Hearts that did not respond to tetanic stimulation, or did not demonstrate stable developed pressures during successive tetanizations under identical conditions of coronary perfusion pressure, temperature, and intraventricular volume, were discarded. At the end of each experiment, the LV was separated from the right ventricular free wall and weighed.

Steady-state pressure-volume relationship at different calcium concentrations. In the first series of hearts (n = 7), the features of the pressure-volume curve during steady-state (tetanic) activation of the myocardium were investigated at different $[Ca^{2+}]_o$ values. After the ryanodine perfusion period, perfusate was switched to a 0.3 mM [Ca^{2+}] baseline solution. The flow rate of this baseline solution was set to provide a perfusion pressure of 80 mmHg and was quantified during the experiment by the readout on the calibrated rotary pump (Masterflex 7518–10). $[Ca^{2+}]_o$ reaching the heart was then varied by infusing a 1 M $CaCl_2$ stock solution into the perfusion line at a rate determined to provide the desired final calcium concentration. In this manner, $[Ca^{2+}]_o$ could be arbitrarily, easily, and reproducibly changed. Mixing of the baseline and stock solutions occurred in a small reservoir close to the heart, and the hypertonic $CaCl_2$ infusion never accounted for >1% of the total perfusate flow. $[Ca^{2+}]_o$ values of 3.0 and 10 mM were tested in all experiments and, when stability of the preparation allowed, 0.5 mM was also tested. The heart was allowed to stabilize at each $[Ca^{2+}]_o$ for at least 5 min. At each $[Ca^{2+}]_o$, tetanic pressure was measured at

approximately six different ventricular volumes that were selected such that the end-diastolic pressure before tetanization varied between 0 and 30 mmHg. The peak pressure during tetanus was measured 2 min after each volume change. To monitor the stability of the preparation, select measurements were repeated at different times throughout the experiment.

Steady-state pressure-volume relationship at saturating calcium concentrations. In the second series of hearts ($n = 8$), the characteristics of the pressure-volume curve during steady-state activation and during twitch (nonsteady-state) activation at a saturating $[Ca^{2+}]_o$ were evaluated. Results from the first series of hearts indicated that at $[Ca^{2+}]_o$ values >8 mM, there was no appreciable increase in developed tetanic pressure with increasing $[Ca^{2+}]_o$, suggesting saturation of the myofilament apparatus with respect to calcium. Consequently, $[Ca^{2+}]_o$ was set at 10 mM for this set of experiments. The end-systolic pressures during both tetanus and spontaneous twitches (mean heart rate 48 ± 6 beats/min) were measured at six volumes that spanned a range of end-diastolic pressures between 0 and 30 mmHg. Intraventricular volumes were changed randomly, and stability of the preparation was assessed intermittently as before.

Steady-state pressure-calcium relationships. In the third series of hearts ($n = 8$), the effect of varying $[Ca^{2+}]_o$ on developed pressure and aequorin luminescence during maximal steady-state activation was tested at different volumes. After ryanodine infusion and aequorin macroinjection, $[Ca^{2+}]_o$ was varied between 0.3 and 20 mM (between 6 and 8 values) in each experiment in the same way as described above. For every $[Ca^{2+}]_o$ tested, developed pressures were measured during tetanization at 2 or 3 volumes, corresponding to approximate EDPs of 0, 10, and 30 mmHg.

Measurement of sarcomere length during steady-state activation at different volumes. In a fourth series of hearts ($n = 4$), sarcomere lengths during steady-state activation at different volumes were measured. Ryanodine-perfused hearts were rapidly fixed at volumes corresponding to approximate EDPs of 0 or 30 mmHg ($n = 2$ each) during tetanus by modification of methods developed by Ross et al. (31) for fixing dog hearts in systole. A polyethylene catheter (2-mm ID), attached to a 20-ml syringe, was positioned just above the aortic valve and primed with an arresting solution (2% glutaraldehyde in phosphate buffer). Immediately as tetanization was induced, 10 ml of fixative were injected forcefully by hand within 1 s. An additional 10 ml of 2% glutaraldehyde were injected slowly after the initial rapid fixation. The heart was subsequently immersed in 2% glutaraldehyde and refrigerated until sections for light microscopic examination and sarcomere length determinations were taken. Hearts were kept immersed in glutaraldehyde solution for at least 72 h. Apical, midwall, and basal cross sections of the LV were then removed (such that midwall myofibers were longitudinally cut) and fixed overnight in 10% neutral, buffered Formalin before dehydration and paraffin embedding. Sections (4 μm) were stained with trichrome and examined on a standard light microscope. Groups of ~20 adjacent sarcomeres were identified in the midwall; the total lengths of these adjacent sarcomere segments were measured using an eyepiece micrometer capable of 0.8-μm resolution with a total magnification of ×1,000. Average sarcomere length was then estimated as total segment length divided by the number of sarcomeres within the segment. Such measurements were taken from the three midwall regions in each heart (apical, midwall, and basal), and an average of 10 measurements were made in each region.

Data analysis. For all protocols, pressure and light signals (L) were digitized at a sampling rate of 500 Hz on a computer for off-line analysis. In addition to examining pressure-volume and pressure-calcium relations directly, LVP and LVV were converted into estimates of muscle stress (σ) and strain (ϵ), respectively, by assuming the ventricle was constructed as a thick-walled sphere with uniform muscle properties and with σ assumed to represent the average stress across the wall (21, 25). Thus LVP was transformed into σ based on inner LV chamber radius (r_i), outer LV chamber radius (r_o), LVV, and LV wall volume (LVWV; which, assuming a tissue density of 1 g/ml, was set equal to the LV mass) according to *Eqs. 2, 3,* and *4*

$$r_i = [3\ \mathrm{LVV}/4\pi]^{1/3} \tag{2}$$

$$r_o = [3\ (\mathrm{LVV} + \mathrm{LVWV})/4\pi]^{1/3} \tag{3}$$

$$\mathrm{LVP} = \sigma(r_o^2 - r_i^2)/r_i^2 \tag{4}$$

By substitution and rearrangement, the following equation for stress is obtained

$$\sigma = \frac{\mathrm{LVP}(\mathrm{LVV})^{2/3}}{(\mathrm{LVV} + \mathrm{LVWV})^{2/3} - \mathrm{LVV}^{2/3}} \tag{5}$$

LVV was then related to midwall circumference (C) of the sphere (21, 25): $C = 2\pi[(r_o + r_i)/2]$. C was then converted into a strain value to provide a value of 1 at a reference LVV that corresponded to an EDP of 30 mmHg (reference volume and circumference, V_{ref} and C_{ref}, respectively) and a value of 0 when C corresponded to an LVV of 0 ml (minimal volume and circumference, V_{min} and C_{min}, respectively), according to the following system of equations (where the subscripts "min" and "ref" refer to parameters at V_{min} and V_{ref}, respectively)

$$r_{i,min} = 0 \qquad r_{0,min} = [3\ \mathrm{LVWV}/4\pi]^{1/3} \tag{6a}$$

$$r_{i,ref} = [3\ V_{ref}/4\pi]^{1/3} \qquad r_{o,ref} = [3\ (\mathrm{LVWV} + V_{ref})/4\pi]^{1/3} \tag{6b}$$

$$C_{min} = 2\pi(r_{o,min} + r_{i,min})/2 \tag{6c}$$

$$C_{ref} = 2\pi(r_{0,ref} + r_{i,ref})/2 \tag{6d}$$

and

$$\epsilon = (C - C_{min})/(C_{ref} - C_{min}) \tag{6e}$$

Active pressure and stress in the heart were calculated as the total pressure or stress at the peak of contraction at a particular volume minus the diastolic pressure or stress at the same volume.

To account for minor variations in absolute levels of contractile strengths between hearts, values of pressure and stress were normalized in two ways. First, these data were expressed as a percentage relative to their respective values at saturating levels of calcium and maximum strain of 1.0; these values will be denoted by the symbol $\%_{rel}$. Second, data at each $[Ca^{2+}]_o$ were normalized to their respective values measured at the reference strain of 1.0; these values will be denoted by the symbol $\%_{norm}$.

In all studies, the steady-state pressure was defined as the peak pressure attained during the tetanus. The corresponding steady-state $[Ca^{2+}]_i$ during the tetanus was defined as the average $[Ca^{2+}]_i$ during the plateau phase in the pressure signal.

The entire analysis was performed using custom-made software (programmed in Microsoft BASIC). Statistical computations, detailed below, were performed using commercially available software (STATVIEW 4.0, Berkeley, CA; SYS-

TAT, Evanston, IL). In all cases, pooled values are means ± SD, and P values < 0.05 were considered statistically significant.

RESULTS

Pressure and Aequorin Luminescence Tracings During Tetani

Typical aequorin light (*top*) and pressure (*bottom*) recordings obtained during the first ~3 s of tetanic contractions at three different $[Ca^{2+}]_o$ values at the same volume (1.10 ml) are shown in Fig. 1. The corresponding calculated $[Ca^{2+}]_i$ tracings (fast-Fourier transform-filtered at a cutoff frequency of 10 Hz) determined from the aequorin signals are shown in the middle panels. At $[Ca^{2+}]_o$ values of 2.5 and 10 mM, distinct light and calcium transients are observed during the individual spontaneous twitch contractions (*arrows*) with reasonable signal-to-noise ratios despite the fact that signal averaging was not employed. The light signals were bright, reaching values of 2 nA on twitches and approaching 8 nA during tetani. $[Ca^{2+}]_i$ signals reached plateau levels 1.5–2 s after initiation of rapid pacing. During the tetani, there were slight oscillations in the pressure signals, which correlated with individual spikes in the aequorin signal; the fluctuations of light were timed precisely to the frequency of rapid pacing. The magnitude of the light fluctuations and pressure were more related to the time after ryanodine exposure (increasing with time after exposure) rather than to the $[Ca^{2+}]_o$. These features of the light signal during twitches and tetani were less apparent with low $[Ca^{2+}]_o$ ($\leq$1 mM).

There was a progressive increase in the plateau value of aequorin light and pressure as $[Ca^{2+}]_o$ was raised from 1.0 to 10 mM. Beyond 10 mM $[Ca^{2+}]_o$, however, the maximal developed pressure during tetani was not appreciably enhanced, whereas the aequorin luminescence, and thus $[Ca^{2+}]_i$, continued to increase (records not shown). This can be taken as evidence that maximal $[Ca^{2+}]_i$-activated pressure had been reached. With release of the tetani, LVP returned rapidly to diastolic levels (tracings not shown). Whereas aequorin luminescence also decreased with release of the tetani, it did not return immediately to baseline value and typically required an average of 15 s (range 4 to 30 s) to return to baseline. This observation is not unique to the present study or to the techniques used to measure $[Ca^{2+}]_i$ and is discussed in detail below.

Steady-State Pressure-Volume and Stress-Strain Relations

Steady-state tetanic pressure-volume relations were measured at different $[Ca^{2+}]_o$ values (Fig. 2*A*). Results are expressed as $\%_{rel}$, a percent of the tetanic pressure obtained in each heart at the highest volume tested (i.e., at an EDP of 30 mmHg) and a $[Ca^{2+}]_o$ of 10 mM. The average absolute LVP measured under these conditions was 289 ± 20 mmHg, which is the same as that measured previously in tetanized ferret hearts (29). Mean LV mass was 3.75 ± 0.32 g, which is also similar to the mass of hearts used in the previous study of ferret hearts (29). A reasonably linear pressure-volume relationship was present at each $[Ca^{2+}]_o$. As $[Ca^{2+}]_o$ was increased, the relation shifted upward and demonstrated a marked reduction in slope. With $[Ca^{2+}]_o$ of 0.5 mM, tetanic pressure decreased by 48% over the volume range tested, whereas with a saturating $[Ca^{2+}]_o$ of

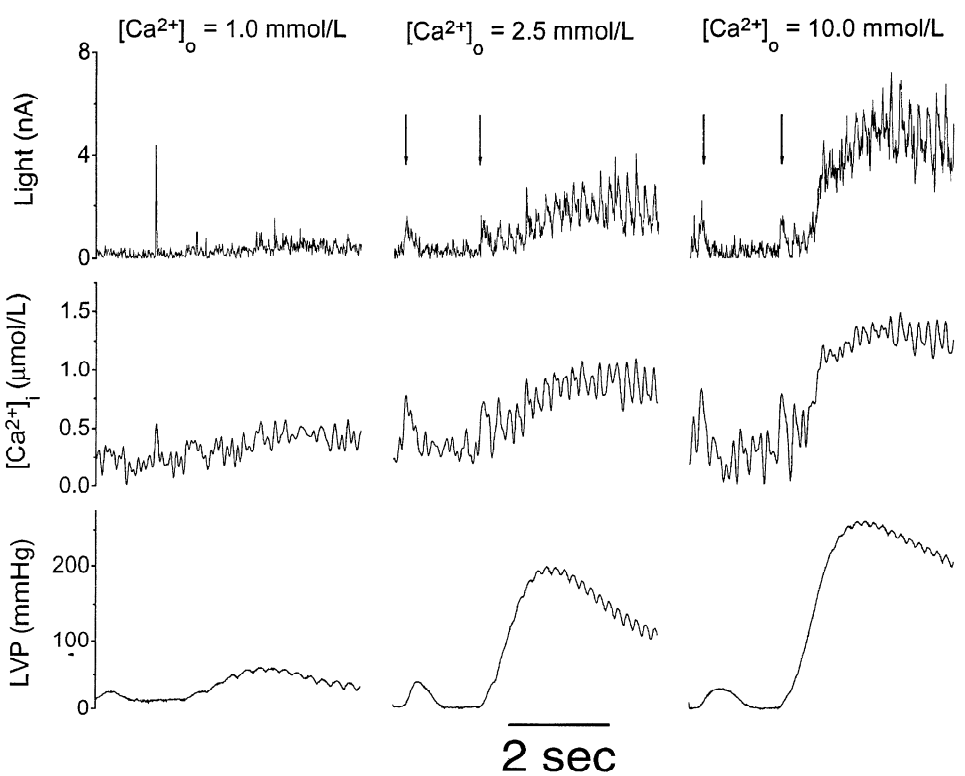

Fig. 1. Original recordings of aequorin light, estimated intracellular calcium concentration ($[Ca^{2+}]_i$), and left ventricular pressure (LVP) at 3 different perfusate calcium concentrations ($[Ca^{2+}]_o$) during tetanic contractions (~3 s of tetanus shown). Aequorin luminescence tracing are not signal averaged. Corresponding estimated $[Ca^{2+}]_i$ tracings are filtered at 10 Hz. *Arrows*: calcium transients during individual spontaneous twitch contractions occurring before tetani. Note that LVP, aequorin luminescence, and $[Ca^{2+}]_i$ during tetanus increase as $[Ca^{2+}]_o$ is raised.

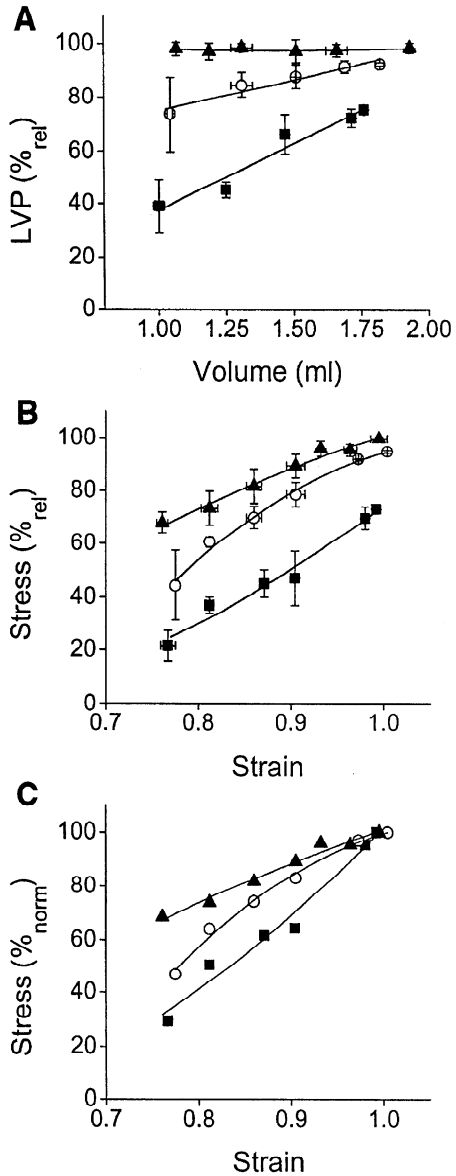

Fig. 2. Average steady-state pressure-volume and stress-strain relations at 3 different $[Ca^{2+}]_o$ (10 mM, ▲, n = 7; 3 mM, ○, n = 6; 0.5 mM, ■, n = 4). Data points were pooled according to strain intervals of 0.025. Data are means ± SD. Whereas steady-state pressure (LVP) at a saturating $[Ca^{2+}]_o$ of 10 mM (*A*) did not vary significantly with volume, relative stress-strain relation was considerably steeper at same $[Ca^{2+}]_o$ (*B*). *C*: data from *B* after normalization (error bars omitted for clarity). Note curves obtained at different $[Ca^{2+}]_o$ values do not superimpose (see text for details).

10 mM, peak pressure did not vary significantly with volume. The curve measured at a $[Ca^{2+}]_o$ of 3 mM was between these two extremes.

The corresponding stress-strain relations are shown in Fig. 2*B*. The range over which LVV was varied resulted in strains that spanned 1.00 (EDP = 30 mmHg) to 0.75 (EDP = 0). As in Fig. 2*A*, the data are expressed as $\%_{rel}$, a percentage of the tetanic stress obtained in each heart at a strain of 1 and a $[Ca^{2+}]_o$ of 10 mM. The average muscle stress measured under these conditions was 236 ± 31 mmHg, which corresponds to 3.21 ± 0.42 g/mm^2. The stress-strain relations using the thick-walled sphere model were steeper than the pressure-volume relations, which is a reflection of the nonlinear nature of the system of equations used to estimate stresses and strains (*Eqs. 2-6*). Thus, whereas tetanic pressure did not vary with volume at 10 mM $[Ca^{2+}]_o$, there was a 33% decrement in stress when strain was decreased from 1.0 to 0.75. The decrements in stress were greater at the lower calcium concentrations. In addition, there was a slight nonlinearity in the relationships that, in the case of the curve measured at $[Ca^{2+}]_o$ of 3 mM, achieved statistical significance by nonlinear least squares analysis. The length dependence of calcium activation is further clarified in Fig. 2*C*, where stress values are now normalized ($\%_{norm}$) to the values obtained at a strain of 1 for the respective $[Ca^{2+}]_o$. These curves indicate that changes in muscle strain had a greater effect on steady-state stress at lower calcium concentrations.

Steady-State and Twitch Activation at Saturating $[Ca^{2+}]_o$

Isovolumic twitch and tetanic pressure-volume and stress-strain relations were determined at 10 mM $[Ca^{2+}]_o$ (Fig. 3). The developed pressures during tetanus were 2.5 to 5 times greater than pressures generated during twitches. As described above, pressure did not vary with volume during tetanic contractions, whereas twitch pressure decreased by 60% over the volume range studied (Fig. 3*A*; data expressed as $\%_{rel}$, percent pressure developed during tetanic contraction measured at an EDP of 30 mmHg). The stress-strain relations during tetanus, as described above, reveal an ~30% reduction in stress when strain is decreased from 1.0 to 0.75 (Fig. 3*B*). Peak twitch stress was significantly lower than during tetanus and was influenced to a much greater degree by volume changes as revealed in Fig. 3*C*, where values have been normalized ($\%_{norm}$) to their respective peak systolic stress measured at a strain of 1. Note the similarities between the relations measured during twitches with $[Ca^{2+}]_o$ of 10 mM (Fig. 3, *B* and *C*) to those measured during tetanic contractions with $[Ca^{2+}]_o$ of 0.5 mM (Fig. 2, *B* and *C*).

Relating $[Ca^{2+}]_o$ to $[Ca^{2+}]_i$

The relationship between $[Ca^{2+}]_o$ and $[Ca^{2+}]_i$ during tetani obtained from all hearts studied is shown by the open circles in Fig. 4. As noted above, $[Ca^{2+}]_i$ during the tetanus was defined as the mean estimated calcium from aequorin luminescence during the plateau of the pressure wave. Also shown in this plot are data reproduced from a previous study in which this same relationship was obtained from isolated ferret papillary muscles that were microinjected with aequorin (29). Despite major differences in the preparations (intact heart vs. intact muscle) and techniques of aequorin injections (macroinjection vs. microinjection), there was considerable overlap between the two data sets, and the relations were indistinguishable [P = 0.97 by analysis of variance (ANOVA)]. Figure 4*B* shows the relationship for data from the present study obtained by averaging $[Ca^{2+}]_i$ values at common $[Ca^{2+}]_o$ values (averages were

taken when there were >2 measurements available at the same $[Ca^{2+}]_o$). An approximate linear relationship is apparent on this log-log plot: $\log[Ca^{2+}]_i = -0.45 + 0.43 \cdot \log[Ca^{2+}]_o$. This indicates a power function relationship between $[Ca^{2+}]_i$ and $[Ca^{2+}]_o$: $[Ca^{2+}]_i = 0.36 \cdot [Ca^{2+}]_o^{0.43}$.

Steady-State Stress-$[Ca^{2+}]_i$ Relation

To directly quantify the volume dependence of calcium activation in the intact heart, pressure-$[Ca^{2+}]_i$ relations were measured at different volumes (corresponding to EDPs of 0, 10, and 30 mmHg). Pressure-volume measurements were then converted into stresses and strains as detailed in METHODS. Data from different hearts were pooled according to strains at values of 1.0, 0.85, and 0.75; note that because data were determined at preset EDP values and not predetermined strain values, linear interpolation was applied to the available data to estimate active stress development at the specified strains. Pooled data exhibited the characteristic sigmoidal relationship between $[Ca^{2+}]_i$ and developed stress seen in isolated muscle. Note that because stress values are expressed as $\%_{norm}$ (a percentage of their respective plateau values), the relationship between $[Ca^{2+}]_i$ and normalized tetanic pressure is identical to the relationship between $[Ca^{2+}]_i$ and normalized stress.

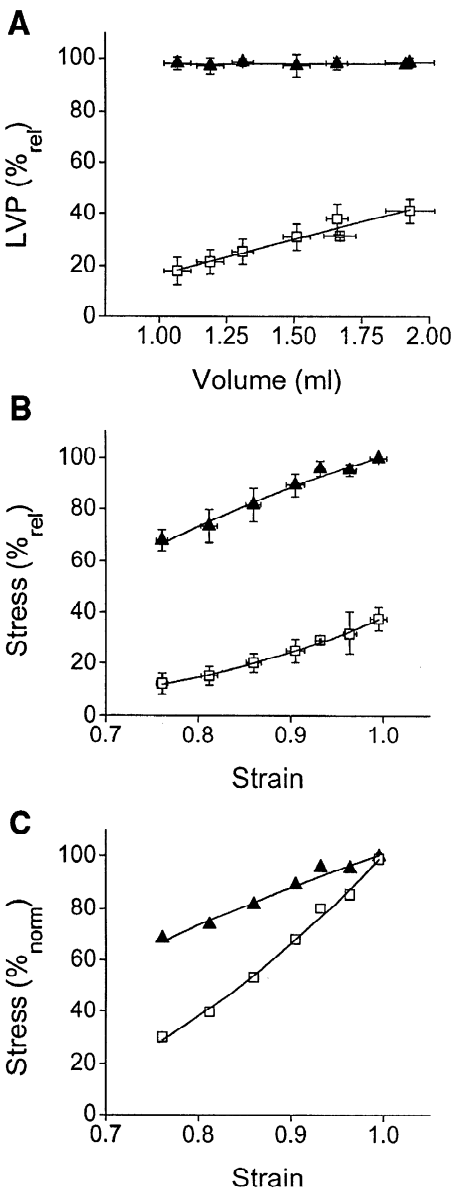

Fig. 3. Average pressure-volume and stress-strain relations during steady-state and twitch activation in 8 hearts at saturating $[Ca^{2+}]_o$ (10 mM). Data points were pooled according to strain intervals of 0.025 and are shown as means ± SD. Peak developed LVP (*A*) and relative stress (*B*) during twitch contractions (□) were much less than during corresponding tetanic contractions (▲). *C*: data from *B* after normalization (error bars omitted for clarity). Note similarities between twitch stress-strain relation at saturating $[Ca^{2+}]_o$ and steady-state stress-strain relation at 0.5 mM $[Ca^{2+}]_o$ (Fig. 2).

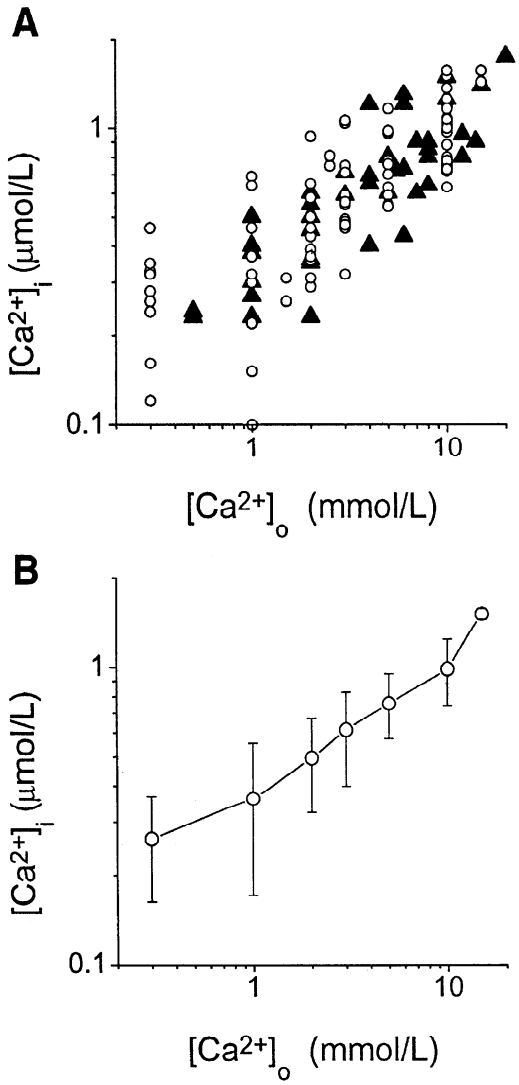

Fig. 4. Relation between $[Ca^{2+}]_o$ and estimated $[Ca^{2+}]_i$ during tetani. *A*: data from previous study in intact ferret papillary muscle [Marban et al. (29); ▲] overlap with data from present study (○; $P = 0.97$). *B*: mean ± SD $[Ca^{2+}]_i$-$[Ca^{2+}]_o$ relationship from present study.

Calcium regulation of cardiac activation was characterized at each strain by a standard Hill equation

$$\sigma = \sigma_{max} \frac{[Ca^{2+}]^{n_H}}{K_{1/2}^{n_H} + [Ca^{2+}]^{n_H}} \quad (7)$$

where n_H is the Hill coefficient, the conventional index of the relative steepness of this relation, and $K_{½}$ is the $[Ca^{2+}]_i$ at half-maximal stress in millimoles. The average Hill relations determined at each strain are shown in the respective panels of Fig. 5; data from each experiment were fit to the Hill equation separately, and then the mean values for n_H and $K_{½}$ were used to construct the relations shown in the figure. The results of this analysis are further illustrated in Fig 6. The mean Hill fits displayed in Fig. 6*A* are the same curves shown in Fig. 5 except that the individual plateau values were set at the percent developed stress ($\%_{rel}$) relative to that measured at a strain of 1.0 and $[Ca^{2+}]_o$ of 10 mM ($[Ca^{2+}]_i \sim 1$ μM). The dotted lines indicate the respective relative stress and $[Ca^{2+}]_i$ values at 50% maximal activation. This set of curves demonstrates the combined effects of length on apparent calcium binding affinity ($K_{½}$) and on maximal force-generating capacity. The same data, now normalized to the maximum stress obtained at the respective strains (Fig. 6*B*, same curves as in Fig. 5), demonstrate the effect of length on apparent calcium binding affinity ($K_{½}$) and also reveal the change in steepness of the curves at decreasing levels of strain. These results are summarized quantitatively in Table 1. It is noted that there is considerable overlap of data points at the different strain values. To determine the statistical significance of changes in both n_H and $K_{½}$, the mean parameter values were compared using ANOVA and a post hoc Bonferroni-Dunnett correction. This analysis revealed a statistically significant difference in both $K_{½}$ and n_H values between strain values of 0.75 and 1.0; values obtained at a strain of 0.85 were not statistically different than those at strains of either 0.75 or 1.0.

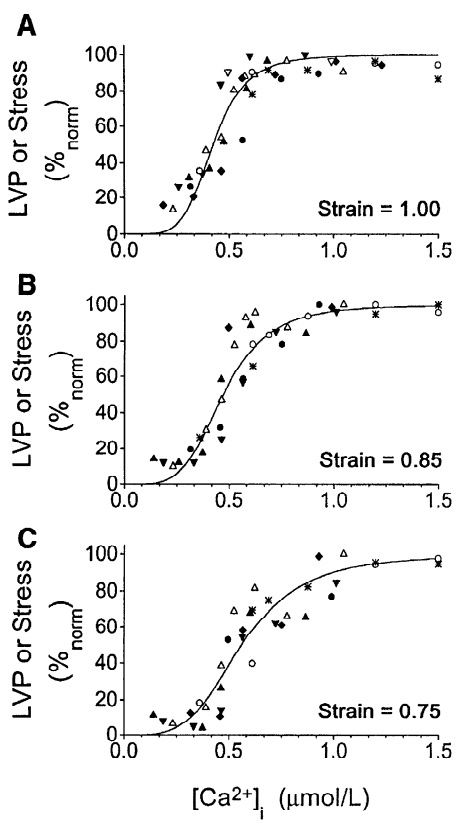

Fig. 5. Normalized steady-state stress (or pressure)-$[Ca^{2+}]_i$ relations from 8 hearts at 3 different strains (*A*, 1.00; *B*, 0.85; *C*, 0.75). Each preparation represented by individual symbol, and Hill fits shown were obtained by averaging best-fitting curves from individual data sets.

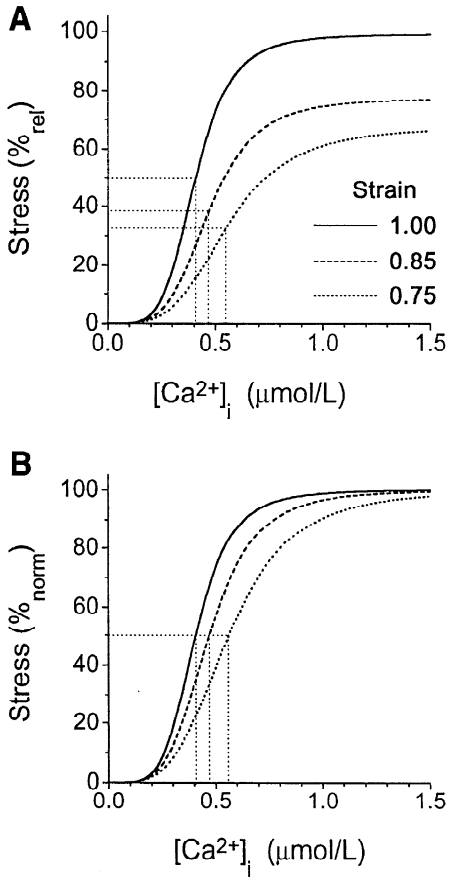

Fig. 6. Average steady-state stress-$[Ca^{2+}]_i$ relations from 8 hearts in Fig. 5. *A*: data shown as percent relative to respective values at strain = 1.0 and saturating calcium levels. Curves reveal that plateau value increases as strain is increased. *B*: data are normalized to maximum values at respective calcium concentrations. $[Ca^{2+}]_i$ to attain 50% maximal stress increases and relative steepness of curve decreases as strain is decreased (see text for details).

Relationship Between Ventricular Strain and Sarcomere Length

To determine the range over which sarcomere lengths vary between the different volumes studied and the

Table 1. *Parameters describing relationship between $[Ca^{2+}]_i$ and contractile strength at different lengths*

Strain	n	P_{max}, mmHg	σ_{max}, g/mm²	$K_{1/2}$, μM	n_H
1.00	5	235 ± 25	2.72 ± 0.50	0.41 ± 0.03	4.91 ± 0.29
0.85	5	231 ± 25	2.10 ± 0.46*	0.47 ± 0.09	4.39 ± 0.93
0.75	6	230 ± 19	1.83 ± 0.52*	0.56 ± 0.07*	3.87 ± 0.69*

Values are means + SD; n, number of experiments. P_{max} and σ_{max}, absolute value of left ventricular pressure and stress, respectively, generated with calcium saturation; $K_{1/2}$, intracellular calcium concn ($[Ca^{2+}]_i$) to achieve half-maximal activation; n_H, Hill coefficient. *$P <$ 0.05 compared with value obtained at a strain of 1.00 by analysis of variance with Bonferroni-Dunnett post hoc test.

degree of sarcomere length variability present in the intact ferret hearts, four hearts were fixed rapidly during tetanus: two hearts at each of the two EDPs investigated. Average sarcomere lengths were determined from the midwall of each section examined, because it was only at the midwall that long strands of myofibrils (≥20 consecutive sarcomeres), which are required for estimation of sarcomere length with light microscopy, could be reliably observed in these hearts. Average sarcomere lengths were 1.86 ± 0.16 μm at an EDP of 0 mmHg (strain ~0.75) and 2.18 ± 0.23 μm at an EDP of 30 mmHg (strain = 1.0). The differences between values obtained at EDPs of 0 and 30 mmHg were statistically significant (unpaired t-test, $P < 0.01$). There was considerable variation in sarcomere lengths observed in each heart, as evidenced by the relatively large standard deviations about the means. However, the degree of variability did not vary with LVV.

DISCUSSION

Ventricular pump properties have been traditionally described in terms of phenomenological theories (20, 32) that bear relatively little relationship to the basic principles of cardiac muscle physiology. Recently, there have been theoretical and experimental efforts made to move toward more mechanistic descriptions of whole heart function (5, 15). This stems in part from the desire to explain a wide range of load-dependent phenomena observed in intact hearts that are not explained by the previous theories (6, 7, 13, 14, 24, 26, 34, 35) and in part from the desire to apply the vast knowledge base obtained from cellular and molecular studies of myocytes to better explain heart function in health and disease. The present study, which explored fundamental interrelationships between volume, pressure generation, and $[Ca^{2+}]_i$ in the intact heart, represents another step toward this goal. The focus was on testing how ventricular volume impacts on calcium responsiveness of the myocardium under steady-state (tetanic) conditions.

The method used to create steady-state contractions (ryanodine tetanization) has been used previously in both isolated intact muscle (36) and in whole heart preparations (29). This technique has gained wide acceptance as a means to study myofilament contractile properties under a state of constant calcium activation. Aequorin macroinjection techniques were used simultaneously to measure $[Ca^{2+}]_i$ (28). Although there are limitations of this method (discussed below), comparison with previous results (29) indicates that the macroinjection technique provides similar $[Ca^{2+}]_o$-$[Ca^{2+}]_i$ relations as those obtained with the aequorin microinjection technique in papillary muscle preparations during tetani studied under similar conditions (Fig. 4). Based on this finding, we contend that the macroinjection technique provides a reasonable estimate of $[Ca^{2+}]_i$ during tetanic contractions.

Mechanisms underlying the length dependence of cardiac muscle contractile force have traditionally been broadly grouped into two categories: *1*) length dependence of calcium activation and *2*) length-dependent physical factors affecting force production. Physical factors, which include such things as length-dependent changes in myofilament overlap, intermyofilament spacing (30), and restoring forces associated with intra- and extracellular matrix components, have been distinguished experimentally from factors related to changes in calcium activation by bathing skinned cardiac muscles with high (saturating) calcium concentrations. These studies have indicated that even with calcium saturation, force production decreases by 30–40% when sarcomere length is decreased from 2.3 (L_{max}) to 1.75 μm ($0.75 \cdot L_{max}$). In the present study, tetanic contractions created while perfusing with calcium concentrations that provided saturating levels of $[Ca^{2+}]_i$ revealed that steady-state pressure did not vary with ventricular volume (Figs. 2 and 3), which was at first a surprising observation. However, when pressure and volume were transformed into stress and strain using a thick-walled spherical model it was found that as volume was decreased from a value that provided an EDP of 30 mmHg (defined as a strain of 1.00) to a volume that provided an EDP of 0 mmHg (which corresponded to a strain of 0.75), stress decreased by ~33%. These data, therefore, reveal a good quantitative correlation between previous results obtained in isolated skinned muscle and the present results obtained in intact hearts regarding the contribution of physical factors to the length dependence of force generation.

Also, as in isolated skinned muscle, the steady-state stress-strain relationship was influenced greatly by $[Ca^{2+}]_o$. As $[Ca^{2+}]_o$ was progressively reduced below the level that provided saturating levels of $[Ca^{2+}]_i$, that relationship became steeper, indicating that peak stress was more dependent on volume (Fig. 2*B*). This point was further amplified when the curves were compared after normalizing each to its respective stress value at a strain of 1.0 (Fig. 2*C*). The fact that these normalized curves do not overlap provides evidence that muscle length (either primarily or secondarily) modulates calcium responsiveness; classically this has been considered to reflect the notion that with nonsaturating levels of $[Ca^{2+}]_i$, calcium binding may decrease with muscle length and this contributes to the observed decrease in force generation. Most recently, however, evidence has been obtained in skinned muscle that suggests that rather than length-dependent changes in calcium binding to myofilaments, changes in interfilament spacing may be the mechanism responsible for length-dependent changes in calcium responsiveness (30). Although the present results do not address the issue of mechanisms underlying length dependence of activation, they do provide the first confirmation that in general, these phenomena are important in the intact whole heart.

Myofilament responsiveness to calcium and its modulation by loading conditions have been most extensively studied by examining the relationship between force generation and $[Ca^{2+}]_i$ during states of steady activation. Major differences in certain aspects of these relations have been reported between intact and skinned cardiac muscles (17, 19, 22, 27, 30, 36). Whereas both

types of preparations exhibit a typical sigmoidal relationship, $K_{½}$ is much lower (by as much as a factor of 10), and n_H is generally greater in the intact compared with the skinned preparations. The results of the present study confirm the sigmoidal relation between $[Ca^{2+}]_i$ and steady-state pressure (and wall stress) generation in the intact heart. As in cardiac muscle, the plateau value of the curves varies with ventricular volume (Fig. 6*A*) because of the physical factors discussed above. Most important, however, was the finding that the average $K_{½}$ and n_H values we obtained at the reference strain of 1.0 (0.41 μM and 4.91, respectively) are very close to those reported for intact muscle contracting at L_{max} [0.5 μM and 6.0 (36), respectively]; also recall that muscle sarcomere length at L_{max} (2.2–2.3 μm) is very close to the average sarcomere length measured in the intact heart at the reference strain of 1.0 (~2.2 μm).

Studies to determine how muscle length impacts on $K_{½}$ and n_H values have been done in skinned preparations. Those studies have shown that as sarcomere length is decreased from L_{max} to $0.75 \cdot L_{max}$, $K_{½}$ increases (from 3 to 6 μM) and n_H decreases (from 4.5 to 2.5; Refs. 22, 27, 30), suggesting that myofilament calcium responsiveness and the degree of myofilament cooperativity both decrease. The results of the present study (Figs. 5 and 6) are consistent with these previous observations, although there are significant quantitative differences in parameter values. As noted above, we attribute these quantitative discrepancies to differences between preparations in which sarcolemmal membranes are intact or disrupted. It is noteworthy that the present data provide the first quantitation of how these parameters vary as a function of preload over a range of volumes that would be encountered in normal physiological states and during acute stress (EDPs as high as 30 mmHg) in a preparation with intact cell membranes.

The $[Ca^{2+}]_i$ that provides myofilament saturation with respect to pressure (or stress) generation corresponded to an $[Ca^{2+}]_i$ of ~1 μM at a strain of 1.0, which is similar to values obtained in intact muscles at L_{max} (2, 19, 29, 36). As in skinned muscle, this value increases as muscle strain is decreased to 0.75, a reflection of both the increase in $K_{½}$ and a decrease in n_H (Fig. 6). However, the value to which this number increases (1.23 ± 0.04 μM) is significantly lower than that measured in skinned muscle in which bathing calcium concentration, to achieve myofilament saturation at a sarcomere length of 1.75 μm, can reach as high as 10 μM or higher (22, 27). Thus, as in intact cardiac muscle (2, 29, 36), the $[Ca^{2+}]_i$ that provides peak activation during steady-state contractions is comparable to that attained in normal twitch contractions during which peak muscle stress is much less. This was explored further in the data of Fig. 3, which shows a comparison between steady-state and twitch pressures (and normalized stress values). For the same $[Ca^{2+}]_o$, twitch pressure is much less than steady-state pressure, a fact attributable to the nonequilibrium conditions present during a twitch (36).

The focus of the experiments presented in this report was on determining the degree to which physiological properties of cardiac muscle previously characterized in isolated preparations could be used as a foundation for explaining whole heart function. The importance of investigating this question stems in part from the possibility that properties of isolated muscle may be different than those of unperturbed muscle within the wall of the intact heart. Previous lessons, which revealed major differences between properties of isolated skinned and intact cardiac muscle (19), underscore the need to consider this possibility. However, the fact that *1*) ventricular architecture is complex, *2*) muscle properties may vary over the heart, and *3*) a range of sarcomere lengths exist at any given ventricular volume raises additional concerns that the global expression of average muscle properties will be distorted. If truly significant, then whole heart function could be explained on the basis of muscle properties only with the use of complex models that account for all these factors. On the contrary, the present results reveal a remarkable similarity between steady-state behavior of isolated intact cardiac muscle and the intact heart when the latter is characterized by stress-strain analysis obtained from a simple transformation of pressure and volume.

There are several limitations of the present study that must be considered. First, our overall strategy has been to measure global ventricular properties and, from these, estimate quantitative information about average muscle properties. Whereas ventricular pressure and volume are considered valid global expressions of average muscle stress and strain, calcium has been measured from only one small region of the heart. Although not investigated under steady-state conditions, calcium transients during spontaneous twitches have been shown previously to be relatively homogeneous when measured over the epicardial surface of the heart (33), providing some basis for the present approach. Nevertheless, the existence of a significant dispersion of $[Ca^{2+}]_i$ values during tetanic contractions would influence the conclusions of the present study. Second, we have used a simple thick-walled spherical model to estimate myocardial stresses and strains. It is known that this model provides estimates of stress that are less than other geometrically more complex models, so the average estimated stress (3.21 g/mm^2) is low compared with measurements in isolated papillary muscle. For example, if stress was calculated using a spheroidal ventricular geometry with a major-to-minor semiaxis ratio of 1.5 (29), then a value of 5.8 g/mm^2 would be obtained. The use of the simpler model was necessary in the present study because the more complex models require knowledge of geometrical parameters (wall thickness, major and minor axes) at the different volumes, which were not measurable during the present studies.

Another limitation relates to the use of aequorin and the macroinjection technique. Aequorin is a nonlinear indicator with less sensitivity at low (diastolic) calcium concentrations. Furthermore, although it has been

proposed that macroinjected aequorin is taken up from the extracellular space by myocytes, the finding that aequorin light emission did not return to diastolic levels immediately after ceasing rapid pacing (discussed in RESULTS) raises the possibility that there may be a contribution to aequorin luminescence from sites other than within the cytosol of myocytes. However, this same phenomenon has been observed to varying degrees in previous studies in which ryanodine tetani have been studied in aequorin-macroinjected hearts as well as fura 2-microinjected papillary muscles (2, 16, 18, 19). The major difference between observations made in isolated muscle and our observations in intact hearts is that in isolated muscle the delayed decay of calcium following release of a tetanus is typically accompanied by a delayed decay of the force. In contrast, LVP returns to baseline values immediately following release of the tetanus. It is possible that cells that take up aequorin in the macroinjected region could also exhibit this simultaneous delay in mechanical relaxation, but because these account for only a small portion of the total number of the cells in the heart, there is no impact on global pressure. Whereas there are limitations of using macroinjected aequorin for quantifying diastolic calcium during periods of tetani, our aim in the present study was to estimate $[Ca^{2+}]_i$ during tetanic contractions. The similarity between the $[Ca^{2+}]_o$-$[Ca^{2+}]_i$ relationship during tetani measured in the intact heart and that measured previously in isolated muscle microinjected with aequorin provides some validation of the measurements under these specific conditions.

In summary, the load dependence of myofilament calcium responsiveness and physical factors that underlie the Frank-Starling mechanism have been characterized and quantified for the intact heart for the first time. The present study provides fundamental information regarding whole heart function. First, the interrelationships between ventricular volume, pressure, and $[Ca^{2+}]_i$ are similar to comparable measurements made in intact isolated muscle when the former are expressed in terms of mean stresses and strains. This is important because it supports the idea that whole heart function can be thought of in terms of sarcomere function without the need for complex models to account for intricacies of cardiac ultrastructure or regional inhomogeneities. This conclusion is consistent with that of previous studies showing that these structural factors do not distort the global expression of other aspects of myocellular function (8–12). Second, the values of parameters that characterize these interrelationships and, in particular, their variation with ventricular volume, have been determined for the first time. This knowledge is critical for taking the next step in developing a means of characterizing whole heart function based on theories of cardiac muscle contraction. The present findings suggest that it will be possible to achieve this goal.

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