REGULAR PAPER

J. Physiol. Sci. Vol. 56, No. 3; Jun. 2006; pp. 219–226
Online Jun. 3, 2006; **doi:10.2170/physiolsci.RP003706**

Use of the Ca-Shortening Curve to Estimate the Myofilament Responsiveness to Ca^{2+} in Tetanized Rat Ventricular Myocytes

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Abstract: We previously estimated the myofilament responsiveness to Ca^{2+} in isolated intact ventricular myocytes, using the steady-state relationship between cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) and cell-shortening during tetanus (Ca-L trajectory). This method was useful and easy; however, it could not be used for a high dose of Ca sensitizer because the instantaneous plots after the application of Ca sensitizer did not make a fixed point of shortening (we used 5% shortening). Therefore we must produce another method to investigate Ca^{2+} responsiveness. For an estimation of a wider range of the Ca-L trajectory, we fitted the Ca-L trajectory data with the Hill equation to construct the Ca-shortening curve. To fit this curve, we measured the maximal shortening, which was on average 31.6%. The value of $[Ca^{2+}]_i$ to produce the half-maximal shortening (Ca_{50}) was dose-dependently decreased by EMD57033 (sensitization). Either isoproterenol or 3-isobutyl-1-methylxanthine increased Ca_{50} (desensitization) with a concomitant increase in intracellular c-AMP. EMD57439, a selective PDE-III inhibitor, did not significantly increase the c-AMP concentration and produced little change in Ca_{50}. These results are in agreement with previous reports with skinned or intact multicellular preparations. The Ca-shortening curve constructed in intact cardiac myocytes can be used to estimate the myofibrillar responsiveness to Ca^{2+} in a wide range of $[Ca^{2+}]_i$.

Key words: calcium (cellular), contractile function, inotropic agents, myocytes, second messengers.

The change in the myofibrillar responsiveness to Ca^{2+} is one of the key mechanisms in the regulation of cardiac contraction [1]. Myofibrillar responsiveness to Ca^{2+} has been investigated most extensively by the use of skinned cardiac muscles [2], in which the sarcolemmal membrane is removed either chemically or mechanically. In skinned preparations, however, intracellular signal transduction pathways that regulate myofibrillar responsiveness to Ca^{2+} might be altered. Therefore it is important to estimate the myofibrillar responsiveness to Ca^{2+} by using intact preparations.

One approach in intact multicellular preparations is the use of tetanic contraction, which produces a quasi-steady state relation between force and $[Ca^{2+}]_i$ [3, 4]. However, this approach may bring on methodological difficulties, such as an inhomogeneous activation of force and limited drug permeation through the tissue. In regard to isolated myocytes, Spurgeon *et al.* [5] proposed the use of the phase-plane diagram of cell length versus intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) during the relaxation of twitch contraction. Equilibrium between cell length and $[Ca^{2+}]_i$, however, may not always be achieved during twitch relaxation, particularly with interventions that increase the decay rate of the $[Ca^{2+}]_i$ transient (e.g., β-adrenoceptor stimulation). Previously, we reported that an instantaneous relation between $[Ca^{2+}]_i$ and cell length (Ca-L trajectory) during tetanus could estimate the relative changes in myofibrillar Ca^{2+} responsiveness in isolated rat ventricular myocytes [6, 7]. For quantitative analysis, we measured the $[Ca^{2+}]_i$ which caused 5% shortening from the resting cell length during the relaxation phase ($Ca_{5\%}$) and calculated the change in $Ca_{5\%}$ from the control level. Although these methods are useful and easy, they cannot estimate the Ca^{2+} responsiveness of instantaneous plots that did not make a 5% shortening (e.g., a high dose of Ca sensitizer).

In the present study, we report more quantitative information on the steady-state relation between $[Ca^{2+}]_i$ and cell shortening. The present method could provide an improved approach to investigating the physiological and pharmacological modulation of the myofibrillar responsiveness to Ca^{2+} in intact cardiac myocytes. Preliminary reports have appeared in abstract form [8].

Received on Apr 12, 2006; accepted on May 31, 2006; released online on Jun 3, 2006; doi:10.2170/physiolsci.RP003706
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MATERIALS AND METHODS

Cell preparation. Single rat ventricular myocytes were enzymatically isolated from male Wistar rats (250–350 g) as described previously [9]. The investigation conformed with the *Guiding Principles for the Care and Use of Animals in the Field of Physiological Sciences* published by the Physiological Society of Japan (19 December 1988). Briefly, the rats were anesthetized with sodium pentobarbitone (15–20 mg/kg, i.p.) and the hearts excised; the aorta was then cannulated to mount the heart on the Langendorff apparatus. After Langendorff perfusion for 3–4 min at 37°C with Ca^{2+}-free Tyrode's solution containing 0.2 mg/ml collagenase (Nitta Zerachin, Tokyo, Japan) and 0.04 mg/ml protease (type XIV, Sigma, St. Louis, MO, USA), the enzymes were washed out with 0.2 mM-Ca^{2+} Tyrode's solution, and the cells were separated from the ventricular tissue. The isolated cells were stored in 1 mM-Ca^{2+} Tyrode solution at 4°C until use (up to 8 h).

Measurement of $[Ca^{2+}]_i$. The myocytes were incubated in 1 mM-Ca^{2+} Tyrode's solution with fura-2 AM (final concentration 4 μM for 10 min at 22°C), the cells were then centrifuged, and the dye-containing solution was removed to stop further dye loading. It was previously shown that ~85% of fura-2 fluorescence was derived from the cytoplasm, with a minor component (~15%) from the intracellular organelles, possibly the mitochondria [6]. Dye-loaded cells were resuspended in 1 mM-Ca^{2+} Tyrode's solution without the dye and placed in a chamber mounted on the stage of an inverted microscope (Diaphot, Nikon, Tokyo, Japan). The excitation and detection of fluorescence from the preparations were essentially the same as described previously [9]. The myocytes were illuminated alternately at 340 nm and 380 nm at 400 Hz using an epifluorescence system (CAM-230, JASCO, Tokyo, Japan). The resultant fluorescence signals were passed through a 500 ± 20 nm band-pass filter before detection with a photomultiplier tube (R268, Hamamatsu Photonics, Hamamatsu, Japan). After each experiment, the background fluorescence was measured from a myocyte without fura-2 loading and with a dimension (length × width) similar to that used for the fluorescence recording. The major component (approximately 70%) of the background fluorescence was instrumental stray fluorescence, and the remainder was cell autofluorescence. There was no significant change in the background fluorescence measured from myocytes without fura-2 loading during the course of tetanic contraction experiments. The ratio of the fluorescence intensities excited at 340 nm and 380 nm ($R = F[340]/F[380]$) was calculated after the subtraction of the background fluorescence.

The values of $[Ca^{2+}]_i$ were calculated with the standard equation [10]:

$$[Ca^{2+}]_i = K_d \times \beta(R - R_{min})/(R_{max} - R). \quad (1)$$

We used the K_d value of 0.24 μM, the R_{min} value of 0.292, and the R_{max} value of 7.43 estimated in previous studies [7, 11]. The third parameter, β, was estimated to be 8.95 [7, 12].

Measurement of cell length. To monitor the changes in cell length and fluorescence signals simultaneously, myocytes were transilluminated with long-wavelength light (>600 nm), and the cell image was projected onto a linear, 612-element photodiode array. Both edges of the myocyte were detected, and the distance between the edges was automatically measured with an edge detector (MOPS-SPL-46A001, Hamamatsu Photonics). The fluorescence and cell length signals were low-pass filtered (100 Hz) and averaged (1–4 sweeps), using a data collection program on a 486 computer (PC-9801 BX, NEC, Tokyo, Japan).

To detect maximal shortening, a CCD-camera was used to record images of myocytes. The images of the cell lengths projected on a wide screen were measured each second. All experiments were performed at room temperature (22 × 2°C).

Measurement of cytosolic c-AMP concentration. We measured the c-AMP concentration of myocytes, using the method described by Katano and Endoh [13] with a slight modification. The cells were incubated with one of the test compounds in Tyrode's solution for 10 min. The cell suspension was then centrifuged (500 rpm for 40 s), and the supernatant was discarded. Cellular c-AMP was extracted by the addition of 500 μl of 0.2 N HCl into test tubes placed in a boiling water bath for 3 min. After centrifugation at 3,500 rpm for 10 min, the c-AMP content of the supernatant was assayed in duplicate, using an enzyme-immunoassay method (cyclic AMP enzyme immunoassay kit, Assay Designs, Inc., Ann Arbor, MI, USA). Concentrations of c-AMP were expressed as picomoles per milligram of protein (pmol/mg protein), measured with Lowry's method [14, 15] and bovine serum albumin as the standard.

Solutions and chemicals. The composition (in mM) of Tyrode's solution was NaCl, 136.9; KCl, 5.4; $MgCl_2$, 0.5; NaH_2PO_4, 0.33; HEPES, 5; glucose, 5; and pH was adjusted to 7.40 × 0.05 with NaOH at 22°C. The $[Ca^{2+}]$ of Tyrode's solution was adjusted by the addition of a small amount of a $CaCl_2$ stock solution (1 M). All chemicals were reagent grade: fura-2 AM (Molecular Probes, Eugene, OR, USA); thapsigargin (Calbiochem, La Jolla, CA, USA); FCCP, ionomycin, isoproterenol (Iso) and 3-isobutyl-1-methylxanthine (IBMX) (Sigma Chemical, St. Louis, MO, USA); EMD57033 [EMD(+)], the (+) enantiomer of the racemic cardiotonic agent EMD53998 and EMD57439 (EMD[–]), the (–) enantiomer of EMD53998, (donated by Pharmaceutical Research, E. Merck, Darmstadt, Germany) [16]. Thapsigargin, EMD(+), and EMD(–) were dissolved in dimethylsulphoxide (DMSO) to make 10 mM stock solution. The final concentration of DMSO was less than 0.3%, which did not influence either fluores-

cence signals or cell shortening. The direct effects of Iso, IBMX, EMD(+), and EMD(–) on the fura-2 fluorescence signals were tested *in vitro* and were negligible at the concentrations used in this study.

Curve fitting and statistical analysis. A nonlinear least-squares fitting of the % shortening data with the Hill curve was carried out with the program Origin (Ver. 5.0J, Microcal Software, Inc., Northampton, MA, USA). The functional form is

$$\%\ \text{shortening} = \max\%S \times ([Ca^{2+}]_i^{nH}/(Ca_{50}^{nH} + [Ca^{2+}]_i^{nH}) \quad (3)$$

where max%S is the maximal % shortening at saturating $[Ca^{2+}]_i$, Ca_{50} is $[Ca^{2+}]_i$ that gives halfmaximal % shortening, and *nH* is the Hill coefficient. Statistical values are given as mean ± SEM. The two-tailed Student's *t*-test was used for statistical comparison with the significance level set at $P < 0.05$.

RESULTS

Steady-state relationship between cytosolic calcium and cell shortening

Myocytes were treated with thapsigargin (0.2 μM) for 10–15 min to inhibit the Ca^{2+} pump of the sarcoplasmic reticulum [17] and activated by a 10 Hz field stimulation for 10 s delivered every minute to induce tetanic contraction. Figure 1, A and B, shows changes in $[Ca^{2+}]_i$ and cell length during tetanus at three different levels of extracellular $[Ca^{2+}]$ ($[Ca^{2+}]_o$) (1, 4 and 8 mM). The cells were bathed for 10 min in each $[Ca^{2+}]_o$ before recording their responses. Both $[Ca^{2+}]_i$ and cell shortening slowly developed during repetitive stimulation and returned to their baselines after the cessation of stimuli. The instantaneous plot of $[Ca^{2+}]_i$ (abscissa) versus % cell-shortening (ordinate) followed a very similar trajectory (Ca-L trajectory, Fig. 2A) during the ascending limb (symbols) and the descending limb (lines). Changes in $[Ca^{2+}]_o$ from 1 to 8 mM extended the trajectory along the same line without any substantial shift of the relation. This trajectory with little hysteresis thus confirmed our previous report [6] and suggested a near equilibrium relation between $[Ca^{2+}]_i$ and cell length during tetanus.

We also estimated the maximal shortening in separate experiments in which cells were repeatedly tetanized at 12 mM $[Ca^{2+}]_o$. In this experiment, myocytes were stimulated every 3 min because of the slow relengthening of the cells after the stimuli. Figure 1C illustrates an example of such an experiment. In this cell, the amplitude of shortening gradually increased from the 1st to the 3rd tetanus. Irreversible changes in the cytoskeleton because of Ca^{2+} overload limited the relengthening of the cells; we analyzed the results from the myocytes that showed a recovery of cell length to more than 90% of the resting length during a 3 min interval. Figure 1D shows the mean amplitude of shortening during the 1st to the 4th tetanus in 6 myocytes. Because the degree of shortening showed a tendency to saturate at the 3rd and 4th contraction, we assumed the maximal degree of shortening (max%S) to be 31.6 ± 1.3% ($n = 6$), measured at the 4th contraction. We found that the maximal degree of shortening estimated in the myocytes was not significantly influenced by EMD(+) (31.0 ± 1.3%), EMD(–) (30.9 ± 2.8%), IBMX (30.9 ± 1.0%), or Iso (31.6 ± 1.3%) ($n = 6$ for each drug). We therefore used the value of 31.6% for max%S for all of the following analyses. A tetanization of myocytes at higher $[Ca^{2+}]_o$ (16 mM) was not successful because of hypercontraction leading to cell death at the first tetanus in all cells tested.

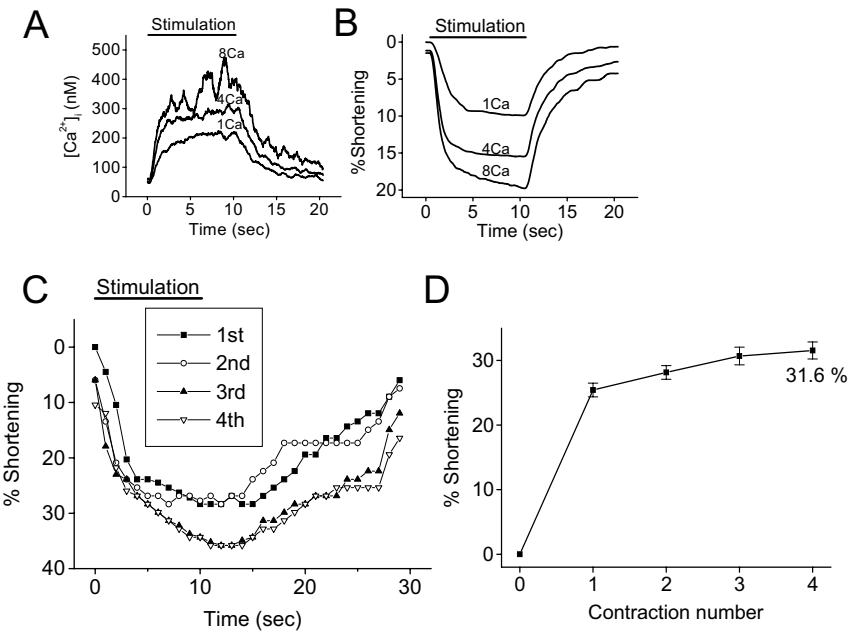

Fig. 1. $[Ca^{2+}]_i$ and cell shortening during tetanus. **A**: Changes in $[Ca^{2+}]_i$ during tetanus at various $[Ca^{2+}]_o$ (labeled in mM). **B**: Changes in cell length during tetanus as shown in A. The degree of shortening is expressed as the % change from the resting cell length. **C**: Changes in the length of a cell during tetanus repeatedly induced every 3 min in 12 mM $[Ca^{2+}]_o$ (1st to 4th indicate contraction number). In A–C, horizontal bars indicate stimulation periods. **D**: Mean value of the maximal cell shortening during tetanus at 12 mM $[Ca^{2+}]_o$ ($n = 6$). Error bar shows SEM. Cell shortening was almost saturated at the 3rd contraction and reached, on average, 31.6% at the 4th contraction.

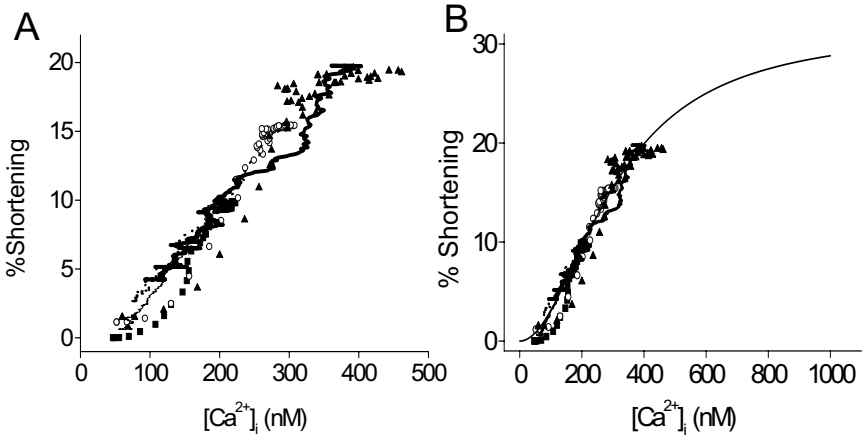

Fig. 2. A: An instantaneous plot of $[Ca^{2+}]_i$ and cell length (Ca-L trajectory) from the data shown in Fig. 1, A and B. Solid squares, an ascending limb of 1 mM $[Ca^{2+}]_o$; open circles, an ascending limb of 4 mM $[Ca^{2+}]_o$; solid triangles, an ascending limb of 8 mM $[Ca^{2+}]_o$. Thin lines, a descending limb of 1 mM $[Ca^{2+}]_o$; broken lines, a descending limb of 4 mM $[Ca^{2+}]_o$; thick lines, a descending limb of 8 mM $[Ca^{2+}]_o$. **B**: The Ca-shortening curve least-squares-fitted to the pooled Ca-L trajectory data ($[Ca^{2+}]_o$ = 1, 4, and 8 mM) is superimposed (a solid line with Ca_{50} of 305 nM and nH of 1.98).

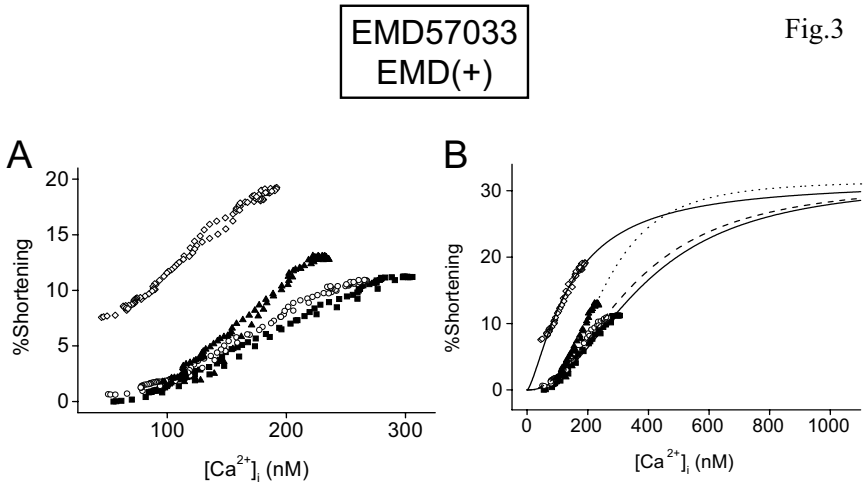

Fig. 3. Effect of EMD(+) on the Ca-L trajectory at 1 mM $[Ca^{2+}]_o$ (symbols in **A** and **B**) and the fitted Ca-shortening curves (lines in **B**). Solid squares and solid line, control; open circles and broken line, 0.3 μM EMD(+); solid triangles and dotted line, 1 μM EMD(+); open squares and solid line, 3 μM EMD(+).

With the average value of the maximal shortening (max%S = 31.6%), the Ca-L trajectory depicted in Fig. 2A could be well fitted by the Hill curve with *nH* of about 2 (Fig. 2B, Ca-shortening curve). There was no significant difference in the fitted parameters for the Ca-L trajectory at three different concentrations of $[Ca^{2+}]_o$. In this and five other myocytes, the mean value of Ca_{50} was 421 ± 49 nM, and the mean value of *nH* was 1.84 ± 0.10. The Ca-L trajectory reached 1/2 to 2/3 of the maximal shortening in these myocytes.

Effect of EMD57033 and EMD57439 on the Ca-shortening curve

The EMD(+) is a Ca^{2+} sensitizing agent that directly affects the cross-bridges [16, 18]. An application of 0.03–3 μM EMD(+) dose dependently decreased the peak $[Ca^{2+}]_i$ and increased the shortening amplitude during tetanus. A high concentration of EMD(+) (3 μM) substantially reduced the resting cell length with a modest decrease in resting $[Ca^{2+}]_i$. Figure 3 shows the effect of EMD(+) on the Ca-L trajectory (A) and the fitted Ca-shortening curve (B). EMD(+) produced a leftward shift of the Ca-L trajectory and the Ca-shortening curve in a dose-dependent manner, which corresponded to an increase in the myofibrillar responsiveness to Ca^{2+}.

On the other hand, EMD(–) is a selective inhibitor of phosphodiesterase (PDE) type-III [16]. It was thought that an inhibition of PDE-III would increase the c-AMP concentration [13, 19] and desensitize the myofilaments by the protein kinase A-dependent phosphorylation of troponin I (Tn-I). In contrast to our expectation, however, EMD(–) (up to 30 μM) did not significantly shift the Ca-L trajectory (Fig. 4A) or the Ca-shortening curve (Fig. 4B).

Figure 5 summarizes the relative changes in Ca_{50} produced by the treatment with EMD compounds. EMD(+) decreased Ca_{50} in a dose-dependent manner, showing a sensitization of the myofilaments; Ca_{50} was significantly decreased to 156 ± 52 nM, from 437 ± 28 nM, (64% reduction) by 3.0 μM EMD(+) ($n = 6$, $p < 0.05$). The value of *nH* was not significantly changed by EMD(+) up to 1.0 μM, but 3.0 μM EMD(+) significantly decreased *nH* to 1.40 ± 0.03, from 1.94 ± 0.23 ($n = 6$, $p < 0.05$). In contrast, EMD(–) up to 30 μM did not significantly alter Ca_{50} or *nH* (Ca_{50} = 449 ± 76 nM and *nH* = 1.96 ± 0.24 in the absence of the drug; Ca_{50} = 445 ± 37 nM and *nH* = 1.91 ± 0.53 in the presence of 30 μM EMD[–] [$n = 6$].

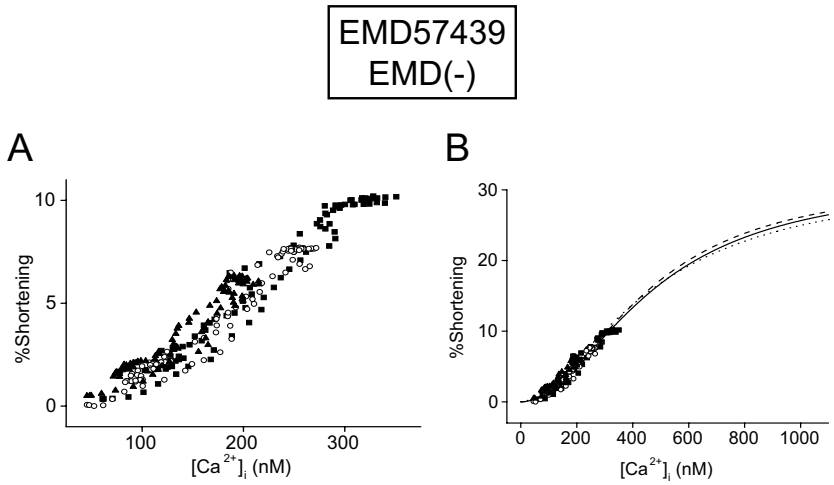

Fig. 4. Effect of EMD(–) on the Ca-L trajectory at 1 mM $[Ca^{2+}]_o$ (symbols in **A** and **B**) and the fitted Ca-shortening curves (lines in **B**). Solid squares and solid line, control; open circles and broken line, 1 μM EMD(–); solid triangles and dotted line, 10 μM EMD(–).

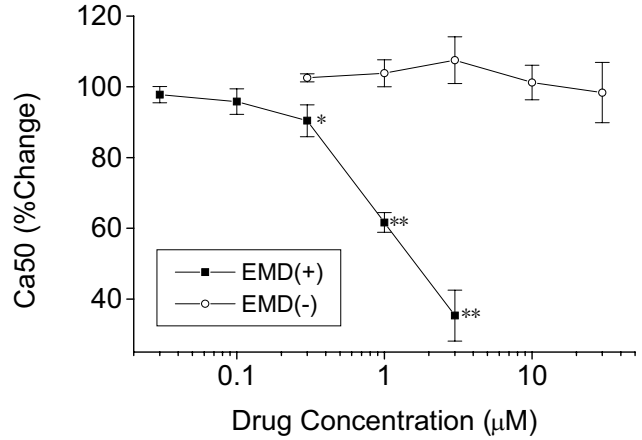

Fig. 5. Effect of EMD(+) (solid squares) and EMD(–) (open circles) on Ca_{50} of the Ca-shortening curve. Relative changes in Ca_{50} from the control value obtained in the absence of any drug were plotted. Error bars show SEM. *p < 0.05 vs. control, **p < 0.01 vs. control.

Effect of IBMX and Iso on the Ca-shortening curve

It is well known that IBMX and Iso increase the c-AMP concentration and that c-AMP–dependent phosphorylation of Tn-I desensitizes the myofibrillar responsiveness to Ca^{2+} [1]. Figure 6 shows the effect of 200 μM IBMX (A) and 10 nM Iso (B) on the Ca-L trajectory and the Ca-shortening curve. It is clear that IBMX and Iso shifted the Ca-shortening curve to the right, which implies a decrease in the myofibrillar responsiveness to Ca^{2+}. The average value of Ca_{50} was significantly increased to 780 ± 96 nM, from 493 ± 28 nM, by 200 μM IBMX (n = 6, p < 0.05), and to 816 ± 106 nM, from 487 ± 35 nM, by 10 nM Iso (n = 6, p < 0.05). The average value of nH was significantly decreased to 1.64 ± 0.11, from 1.94 ± 0.05, by 200 μM IBMX (n = 6, p < 0.05), and to 1.63 ± 0.18, from 1.85 ± 0.17, by 10 nM Iso (n = 6, p < 0.05).

c-AMP concentration

To determine whether the c-AMP concentration was increased by EMD(–), Iso, or IBMX, we measured the c-AMP concentration in myocytes with or without the application of these drugs. The control value of c-AMP concentration from the myocytes with no drug treatment was

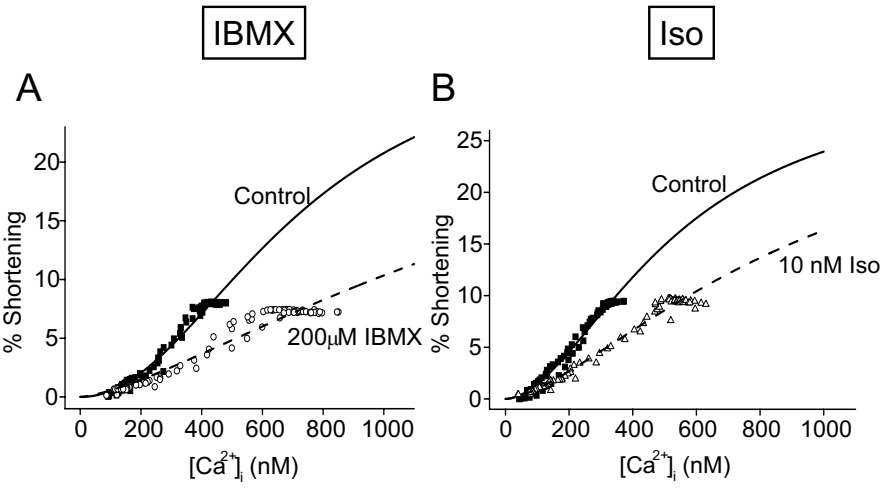

Fig. 6. Effect of IBMX (**A**) and Iso (**B**) on the Ca-L trajectory at 1 mM $[Ca^{2+}]_o$ (symbols) and the fitted Ca-shortening curve (solid and dotted lines). **A**: Solid squares and solid line, control; open circles and dotted line, 200 μM IBMX. **B**: Solid squares and solid line, control; open triangles and dotted line, 10 nM Iso.

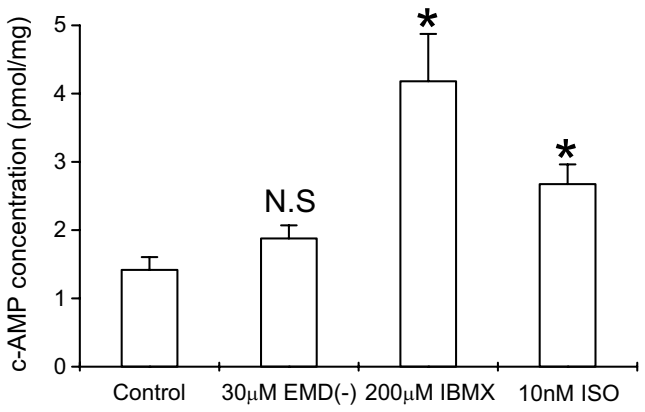

Fig. 7. c-AMP concentration in isolated rat ventricular myocytes treated with various compounds listed at the bottom. Data are expressed as mean ± SEM. (n = 5). *p < 0.05 vs. control.

1.42 ± 0.19 pmol/mg-protein (n = 5). Figure 7 shows that 30 μM EMD(–) did not significantly increase the c-AMP concentration (1.88 ± 0.19 pmol/mg protein, n = 5), whereas IBMX and Iso did significantly increase the c-AMP concentration of myocytes (4.18 ± 0.69 pmol/mg protein with 200 μM IBMX, n = 5, p < 0.05; 2.67 ± 0.29 pmol/mg protein with 10 nM Iso, n = 5, p < 0.05).

The summarized data are shown in Table 1. (changes in Ca_{50}, *nH,* and c-AMP by an application of 30 μM EMD [–], 200 μM IBMX, and 10 nM Iso).

DISCUSSION

Steady state relation between $[Ca^{2+}]_i$ and shortening

The tetanization of myocytes markedly slowed the entire time course of the Ca^{2+} transients so that the relation between $[Ca^{2+}]_i$ and cell shortening was near a steady state, as indicated by the Ca-L trajectory with little hysteresis. An advantage of the present method is that the steady-state relation between $[Ca^{2+}]_i$ and cell shortening could be obtained over a relatively wide activation range (up to 2/3 of the maximum shortening) at one level of $[Ca^{2+}]_o$. Using an estimate of the maximum amplitude of cell shortening, we found that the fitting of the Ca-L trajectory by the Hill equation to construct the Ca-shortening curve extended the relation between $[Ca^{2+}]_i$ and cell shortening to a wider range of $[Ca^{2+}]_i$. The use of Ca_{50} of the Ca-shortening curve, rather than $Ca_{5\%}$ ($[Ca^{2+}]_i$, (which gives a 5% cell shortening) as proposed previously [7], should offer a more sensitive measurement of the myofilament responsiveness to Ca^{2+} because of the greater dynamic range.

The present measurements indicate that $[Ca^{2+}]_i$ is about 30 nM at rest and about 300 nM with cell length shortened by 10%. Further cell shortening to 20% of the resting length showed $[Ca^{2+}]_i$ of about 500–600 nM. The accuracy of these $[Ca^{2+}]_i$ values, however, critically depends on the calibration of fura-2 fluorescence signals. Because it has been reported that the Ca^{2+} affinity of fura-2, as well as other indicators, is very likely to be reduced in the myoplasm as a result of the binding of dyes to intracellular proteins [20], the use of a K_d value, which is estimated in the in vitro solutions, could cause an underestimation of $[Ca^{2+}]_i$ in the present study. For example, if the K_d of fura-2 is 3 times greater in the myoplasm than in the aqueous solutions, as suggested for indo-1 in cardiac myocytes [21], all of the present calculated values of $[Ca^{2+}]_i$ would be underestimated by a factor of three. Because K_d serves as a scaling factor (see Eq. 1), this uncertainty quite likely shifts the Ca_{50} value of the Ca-shortening curve with no changes in *nH*.

In the present study, we determined the maximal degree of cell shortening from the shortest cell length achieved during repeated tetani at 12 mM $[Ca^{2+}]_o$. During cell shortening, the parallel elastic component is compressed, depending on the contractile force. The extent of compression depends on the cell length, and the repulsion force of the elastic component works as a resistance to shortening. In the steady state contraction during tetanus, the contractile force and repulsion force should be equilibrated. The collapse of the elastic component leads to a failure of a relengthening of the cell, or hypercontraction. In our measurements of maximal shortening, we did not include hypercontracted cells that could not relengthen to more than 90% of the resting length. In fact, hypercontracted cells showed a significant shift of the $[Ca^{2+}]_i$-shortening relation, whereas the cells to construct Ca-L trajectory had little shift of the relation as an indication of the maintained elastic component (data not shown). The

Table 1. Changes in Ca_{50} (ΔCa_{50}), *nH* (ΔnH), and c-AMP (Δc-AMP) by an application of 30 μM EMD(–) (n = 6), 200 μM IBMX (n = 6), and 10 nM Iso (n = 5). Data are expressed as mean ± SEM. *p < 0.05, compared with the control. N.S., no significant difference from control.

	ΔCa_{50} (nM)	ΔnH	Δc-AMP (pmol/mg protein)
30 μM EMD(–)	–47.4 ± 39.9$^{N.S}$	–0.0841 ± 0.303$^{N.S.}$	0.837 ± 0.349$^{N.S.}$
200 μM IBMX	383 ± 119*	–0.292 ± 0.116*	5.04 ± 1.11*
10 nM Iso	329 ± 82.6*	–0.225 ± 0.0603*	2.28 ± 0.518*

average value of max%S thus estimated was 31.6%, which was somewhat smaller but in reasonable agreement with the 37% value obtained by Zhou *et al.* [22], who applied a series of long depolarizations under a whole-cell patch clamp. The sarcomere length, which was previously measured in isolated rat ventricular myocytes [6], was on average 1.80 μm at rest. If the sarcomere length changes in parallel with cell length during tetanus, as demonstrated for twitch contraction by Sasaki *et al*. [23], the sarcomere length would decrease to 1.23 μm at the maximal shortening. However, we could not directly measure the minimal sarcomere length in the present study because the striation tended to be unclear during strong contraction, and such a short sarcomere length was difficult to resolve.

Estimation of the myofibrillar responsiveness to Ca^{2+} using the Ca-shortening curve

Changes in $[Ca^{2+}]_o$ did not shift the Ca-shortening curve; however the agents known to alter the Ca^{2+} sensitivity of myofilaments shifted the Ca-shortening curve in the expected direction (Fig. 6). The increase in Ca_{50} and decrease in *nH* by IBMX (200 μM) and Iso (≥10 nM) are attributable to the increased level of intracellular c-AMP by these agents (Fig. 7). The subsequent protein kinase A (PKA)–dependent phosphorylation of Tn-I could then occur by these agents. PKA is also known to phosphorylate myosin-binding protein C. But it is widely accepted that the Ca^{2+} desensitizing effect of myofilaments is mainly due to the phosphorylation of Tn-I [24]. The functional effects of these drugs do not seem to be proportional to c-AMP concentration. Steinberg *et al.* [25] reported that β-adrenergic effects correlated with the activation of the particulate or membrane-bound PKA; an elevation of c-AMP in the soluble fraction and the consequent activation of PKA in the soluble pool were without functional consequences. They also reported that PDE activities were localized to prevent the spread of β-adrenergic signals from particulate locations to soluble fractions, and PDE inhibitor (such as IBMX) reduced this apparent compartmentation. Although we did not measure the phosphorylation level of Tn-I directly, these data support our current results.

On the other hand, EMD(–) did not significantly increase the c-AMP concentration (Fig. 7), and there were no significant changes in Ca_{50} and *nH* (Fig. 4). The lack of any substantial effects of EMD(–) could be due to the relatively weak activity of PDE-III in the rat heart [19]. Concentrations of EMD(–) higher than 30 μM were not tested because of a slight (~1%) contamination of EMD(+) compound in the EMD(–) sample [15]. EMD(+) at 1 μM or lower concentrations reduced Ca_{50} with no obvious changes in *nH*. The value of nH was significantly decreased only with a higher concentration of EMD(+) (3 μM). These results qualitatively agree with the previous reports for the $[Ca^{2+}]_i$-force relation in multicellular preparations [3, 16, 18] and support the validity of the present method.

Comparison of $[Ca^{2+}]_i$-shortening relation with the $[Ca^{2+}]_i$-force relation in multicellular preparations

Although $[Ca^{2+}]_i$-force and $[Ca^{2+}]_i$-shortening relations show qualitatively similar changes when inotropic agents are applied, a direct and quantitative comparison of these relations is rather difficult because the contraction mode of isometric force generation and shortening differs. Cell length is one of the important factors that influences the Ca^{2+} sensitivity of the myofilaments [26–28]. Since the cell length of single myocytes continuously changes during tetanic contraction, the Ca-L trajectory (and the Ca-shortening curve) should be a mixed expression of different Ca^{2+} sensitivity (i.e., multiple Ca-force curves) at different cell lengths. The value of Ca_{50}, $[Ca^{2+}]_i$ at 15.8% cell shortening (i.e., 84.2% of cell length) may reflect the myofilament Ca^{2+} responsiveness at the sarcomere length of, on average, 1.52 μm (1.80 μm × 0.842).

The values of *nH* generally reported for the $[Ca^{2+}]_i$-force relation lie in the range of 3–6 [3, 4, 16, 18, 28], which is thought to reflect cooperativity of the myofilaments. Changes in cell length during tetanus probably contribute to the lower value of *nH* of the Ca-shortening curve (approximately 2). Because cell shortening decreases the Ca^{2+} responsiveness of myofilaments as revealed by the rightward shift of the $[Ca^{2+}]_i$-force relation [3, 18, 28], the Ca-shortening curve should cross over a series of the Ca-force curves as the cell shortens and lengthens, forming a more shallow slope than the Ca-force curve at a fixed length.

Although 3.0 μM EMD(+) has been reported to enhance the maximal force generation by about 10% [15], we could detect no change in the maximal shortening by EMD(+) in the present study. This difference between maximal force and maximal shortening could arise because shortening is not a simple reflection of cross-bridge behavior, and it contains the characteristics of elastic components, whereas isometric force is thought to be a function of the number of cross-bridges and the power of each cross-bridge [29]. Thus care should be taken to interpret any quantitative information on the cross-bridge behavior and cooperativity of myofilaments when based on the $[Ca^{2+}]_i$-shortening relation.

The authors thank Dr. N. Beier (E. Merck) for providing EMD compounds and Miss N. Tomizawa for her technical assistance. Y. Kusakari, K. Hongo, and M. Kawai thank Professor S. Mochizuki, Department of Cardiology, the Jikei University School of Medicine, for continuous encouragement. Part of this study was supported by a Grant-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology (to YK, KH, and SK), by a grant from Uehara Memorial Foundation to YK and SK, and by a grant from Takeda Foundation to KH.

REFERENCES

1. Lee JA, Allen DG. Altering the strength of the heart: basic mechanisms. In: Lee JA, Allen DG, editors. Modulation of cardiac calcium sensitivity. Oxford; Oxford University Press. 1993; p. 1-36.
2. Strauss JD, Rüegg JC, Lues I. In search of calcium sensitizer compounds; from subcellular models of muscle to in vivo positive inotropic action. In: Lee JA, Allen DG, editors. Modulation of cardiac calcium sensitivity. Oxford: Oxford University Press. 1993; p. 37-66.
3. Okazaki O, Suda N, Hongo K, Konishi M, Kurihara S. Modulation of Ca^{2+} transients and contractile properties by β-adrenoceptor stimulation in ferret ventricular muscles. J Physiol (Lond). 1990;423:221-40.
4. Yue DT, Marban E, Wier WG. Relationship between force and intracellular $[Ca^{2+}]$ in tetanized mammalian heart muscle. J Gen Physiol. 1986;87:223-42.
5. Spurgeon HA, duBell WH, Stern MD, Sollott SJ, Ziman BD, Silverman HS, Capogrossi MC, Taro A, Lakatta EG. Cytosolic calcium and myofilaments in single rat cardiac myocytes achieve a dynamic equilibrium during twitch relaxation. J Physiol (Lond). 1992;447:83-102.
6. Hongo K, Kusakari Y, Konishi M, Kurihara S, Mochizuki S. Estimation of myofibrillar responsiveness to Ca^{2+} in isolated rat ventricular myocytes. Pflügers Arch. 1998;436:639-45.
7. Kusakari Y, Hongo K, Kawai M, Konishi M, Kurihara S. The mechanism of increasing Ca^{2+} responsiveness by α_1-adrenoceptor stimulation in rat ventricular myocytes. Jpn J Physiol. 52: 2002;531-9.
8. Hongo K, Kusakari Y, Konishi M, Kurihara S, Mochizuki S. Effect of inotropic interventions on the myofilament responsiveness to Ca^{2+} estimated by tetanus in isolated rat ventricular myocytes (Abstract). Circulation. 1998;98 Suppl:I143.
9. Hongo K, Konishi M, Kurihara S. Cytoplasmic free Mg^{2+} in rat ventricular myocytes studied with the fluorescent indicator furaptra. Jpn J Physiol. 1994;44:357-78.
10. Grynkiewicz G, Poenie M, Tsien RY. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J Biol Chem. 1985;260:3440-50.
11. Berlin JR, Konishi M. Ca^{2+} transients in cardiac myocytes measured with high and low affinity Ca^{2+} indicators. Biophys J. 1993;65:1632-47.
12. Bakker AJ, Head SI, Williams DA, Stephenson DG. Ca^{2+} levels in myotubes grown from the skeletal muscle of dystrophic (mdx) and normal mice. J Physiol (Lond). 1993;460:1-13.
13. Katano Y, Endoh M. Effect of a cardiotonic quinolinone derivative Y-20487 on the isoproterenol-induced positive inotropic action and cyclic AMP accumulation in rat ventricular myocardium: comparison with rolipram, Ro 20-1724, milrinone, and isobutylmethylxanthine. J Cardiovasc Pharmacol. 1992;20:715-22.
14. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the folin phenol reagent. J Biol Chem. 1951;193:265-75.
15. Ohnishi ST, Barr JK. A simplified method of quantitating protein using the biuret and phenol reagents. Anal Biochem. 1978;86:193-200.
16. Lues I, Beier N, Jonas R, Klockow M, Haeusler G. The two mechanisms of action of racemic cardiotonic EMD53998, calcium sensitization and phosphodiesterase inhibition, reside in different enantiomers. J Cardiovasc Pharmacol. 1993;21:883-92.
17. Janczewski AM, Lakatta EG. Thapsigargin inhibits Ca^{2+} uptake, Ca^{2+} depletes sarcoplasmic reticulum in intact cardiac myocytes. Am J Physiol. 1993;265:H517-22.
18. Dubrunz LE, Backx PH, Yue DT. Steady-state $[Ca^{2+}]_i$-force relationship in intact twitching cardiac muscle: direct evidence for modulation by isoproterenol and EMD 53998. Biophys J. 1995;69:189-201.
19. Weishaar RE, Kobylarz-Singer DC, Steffen RP, Kaplan HR. Subclasses of cyclic AMP-specific phosphodiesterase in left ventricular muscle and their involvement in regulating myocardial contractility. Circ Res. 1987;61:539-47.
20. Konishi M, Olson A, Hollingworth S, Baylor SM. Myoplasmic binding of fura-2 investigated by steady-state fluorescence and absorbance measurements. Biophys J. 1988;54:1089-104.
21. Bassani JWM, Bassani RA, Bers DM. Calibration of indo-1 and resting intracellular $[Ca]_i$ in intact rabbit cardiac myocytes. Biophys J. 1995;68:1453-60.
22. Zhou Z, Matlib MA, Bers DM. Cytosolic and mitochondrial Ca^{2+} signals in patch clamped mammalian ventricular myocytes. J Physiol (Lond). 1998;507:379-403.
23. Sasaki N, Mitsuiye T, Noma A, Powell T. Sarcomere length during contraction of isolated guinea-pig ventricular myocytes. Pflügers Arch. 1999;437:804-11.
24. Garvey JL, Kranias EG, Solaro RJ. Phosphorylation of C-protein, troponin I and phospholamban in isolated rabbit hearts. Biochem J. 1988;249:709-14.
25. Steinberg SF, Brunton LL. Compartmentation of G protein-coupled signaling pathways in cardiac myocytes. Annu Rev Pharmacol Toxicol. 2001;41:751-73.
26. Allen DG, Kurihara S. The effects of muscle length on intracellular calcium transients in mammalian cardiac muscle. J Physiol (Lond). 1982;327:79-94.
27. Kentish JC, ter Keurs HEDJ, Ricciardi L, Bucx JJJ, Noble MIM. Comparison between the sarcomere length-force relations of intact and skinned trabeculae from rat right ventricle. Circ Res. 1986;58:755-68.
28. Komukai K, Kurihara S. Length dependence of Ca^{2+}-tension relationship in aequorin-injected ferret papillary muscles. Am J Physiol. 1997l;273:H1068-74.
29. Lee JA, Allen DG. Calcium sensitisers: mechanisms of action and potential usefulness as inotropes (Review). Cardiovasc Res. 1997;36:10-20.