The Mechanism of Increasing Ca^{2+} Responsiveness by α_1 -Adrenoceptor Stimulation in Rat Ventricular Myocytes

Yoichiro KUSAKARI, Kenichi HONGO*, Makoto KAWAI*,
Masato KONISHI†, and Satoshi KURIHARA

Department of Physiology II and *Department of Cardiology, The Jikei University School of Medicine,
Minato-ku, Tokyo, 105–8461 Japan; and

†Department of Physiology I, Tokyo Medical University,
Shinjuku-ku, Tokyo, 160–8402 Japan

Abstract: We investigated the mechanism of α_1 -adrenoceptor stimulation on the myofibrillar Ca^{2+} responsiveness at steady-state in intact rat ventricular myocytes. We produced tetanus, and an instantaneous plot of $[\text{Ca}^{2+}]_i$ vs. cell length (Ca–L trajectory) was constructed to estimate the Ca^{2+} responsiveness. An α_1 -agonist, phenylephrine, dose-dependently shifted the Ca–L trajectory to the left, corresponding to sensitization of the myofilaments. An α_1 -antagonist, prazosin, and inhibition of the Na/H exchange by ethylisopropylamiloride (EIPA) completely reversed the phenylephrine-induced shift. Phenylephrine increased pH_i ($\Delta\text{pH}_i = +0.1$), which was reversed

by prazosin and EIPA. Chelerythrine, an inhibitor of protein kinase C (PKC), completely blocked the effects of phenylephrine on Ca^{2+} responsiveness and pH_i . When pH_i was increased ($\Delta\text{pH}_i = +0.1$) without phenylephrine by changing pH_o , the Ca–L trajectory was shifted to the same extent as that observed with phenylephrine. We conclude that α_1 -adrenoceptor stimulation activates Na/H exchange through a PKC-mediated pathway and that an increase in pH_i is mainly responsible for the increase in Ca^{2+} responsiveness. [Japanese Journal of Physiology, 52, 531–539, 2002]

Key words: Ca^{2+} sensitivity, phenylephrine, Na/H exchange, intracellular pH, protein kinase C (PKC).

It has been reported that α_1 -adrenoceptor stimulation increases contraction with or without a significant change in the peak of Ca^{2+} transient, and an increase in the Ca^{2+} responsiveness of the contractile element is considered to be one of the possible mechanisms [1, 2]. The increase in the Ca^{2+} responsiveness of the contractile element by α_1 -adrenoceptor stimulation is explained by intracellular alkalinization or phosphorylation of the contractile elements or both, but the relative contribution of the two mechanisms is still unclear owing to controversy in experimental results [3–6].

For the assessment of Ca^{2+} responsiveness, a phase–plane loop (trajectory), obtained by measuring

the relation between the intracellular Ca^{2+} transient and cell shortening in a single isolated myocyte loaded with a fluorescent dye, has been widely used [7]. The α_1 -adrenoceptor stimulation shifts the trajectory, in particular the re-lengthening phase, to the left, which suggests an increase in Ca^{2+} responsiveness. However, the re-lengthening phase of the trajectory, particularly in unloaded shortening, is significantly influenced by repulsion (restoring) force of the elastic components including cytoskeleton, connectin, and other factors rather than the active cross-bridges [8]. Therefore, the re-lengthening phase of the trajectory, in particular the later phase, does not directly reflect the Ca^{2+} responsiveness measured using the pCa–ten-

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Correspondence should be addressed to: Yoichiro Kusakari, Department of Physiology II, The Jikei University School of Medicine, 3–25–8 Nishi-shinbashi, Minato-ku, Tokyo, 105–8461 Japan. Tel: +81–3–3433–1111 ext 2221, Fax: +81–3–3433–1279, E-mail: kusakari@jikei.ac.jp

sion relation under isometric conditions, even though both cell length and intracellular Ca^{2+} are apparently equilibrated in the re-lengthening phase. Thus, the estimation of Ca^{2+} responsiveness of the contractile element using the trajectory of re-lengthening in twitch is not an appropriate quantitative measure of Ca^{2+} responsiveness.

We previously reported that the trajectory measured using tetanic contraction in a single isolated myocyte loaded with fura-2 is useful for the estimation of Ca^{2+} responsiveness [9]. In tetanic contraction, the active cross-bridges are formed during sustained contraction and the contractile element is more equilibrated with intracellular Ca^{2+} because the time course of the Ca^{2+} signal is much slower than that in twitch. In addition, the trajectory loop is much narrower than that in twitch. Thus, the estimation of Ca^{2+} responsiveness using the trajectory in tetanic contraction is considered to be much closer to the result obtained using the pCa–tension relation in isometric contraction.

In the present study, we investigated the following issues using the trajectory of tetanic contraction in fura-2–loaded single myocytes: (1) increase in Ca^{2+} responsiveness by α_1 -adrenoceptor stimulation; (2) intracellular mechanism of the increase in Ca^{2+} responsiveness by α_1 -adrenoceptor stimulation, in particular, the involvement of intracellular alkalization; and (3) contribution of Na/H exchange through protein kinase C (PKC) activation to α_1 -adrenoceptor stimulation. We extended the previous method by conversion of fura-2 fluorescence signals to intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) using an *in vivo* calibration method and obtained the relation between $[\text{Ca}^{2+}]_i$ and cell length during tetanic contraction, which was a semi-quantitative measure of the Ca^{2+} responsiveness. We also measured intracellular pH (pH_i) change using 2',7'-bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF), a pH indicator, and confirmed the correlation between an increase in Ca^{2+} responsiveness and cytoplasmic alkalization, both of which were inhibited by an α_1 -adrenoceptor antagonist, a Na/H inhibitor, or a PKC inhibitor. Finally, we examined whether or not the change in pH_i induced by a change in pH in solution could explain the shift of the trajectory delivered by α_1 -adrenoceptor stimulation.

A preliminary report has already appeared in abstract form [10].

MATERIALS AND METHODS

Cell preparation. Single rat ventricular myocytes were enzymatically isolated from male Wistar rats (250–350 g) as described previously [11]. The in-

vestigation conformed to the “Guide for the Care and Use of Laboratory Animals” published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Briefly, rats were anaesthetized with pentobarbital sodium (15–20 mg/kg, i.p.) the hearts were excised, and the aorta was cannulated to mount the heart on a Langendorff apparatus. After Langendorff perfusion for 3–4 min at 37°C with Ca^{2+} -free Tyrode solution containing 0.2 mg/ml collagenase (Nitta Zerachin, Tokyo, Japan) and 0.04 mg/ml protease (type XIV, Sigma Chemical, St. Louis, MO, USA), the enzymes were washed out with 0.2 mM Ca^{2+} -Tyrode solution, and cells were dispersed from the ventricular tissue into a dish. We obtained myocytes from the left ventricle or septum, because α_1 -adrenoceptor could be localized in the left ventricle or septum [12]. The isolated cells were stored in 1 mM Ca^{2+} -Tyrode solution at 4°C until use (up to 8 h).

Fluorescence measurement. Myocytes were incubated in Tyrode solution containing 1 mM Ca^{2+} and fura-2 acetoxymethyl ester (AM) (final concentration 4 μM for 10 min at 22°C), and then cells were centrifuged and the dye-containing solution was removed to stop further dye loading. Dye-loaded cells were resuspended in 1 mM Ca^{2+} -Tyrode solution without the dye and placed in a chamber mounted on the stage of an inverted microscope (Diaphot, Nikon, Tokyo, Japan). Excitation and detection of fluorescence from the preparations were essentially the same as described previously [9]. Myocytes were illuminated alternately at 340 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 bandpass filter before detecting with a photomultiplier tube (R268, Hamamatsu Photonics, Hamamatsu, Japan). After each experiment, background fluorescence was measured from a myocyte without fura-2 loading and with a similar dimension (length $\times$ width) to that used for the fluorescence recording. The ratio of the fluorescence intensities excited at 340 and 380 nm [$R = F(340)/F(380)$] was calculated after subtraction of the background fluorescence.

BCECF was also loaded with the AM ester [13, 14]. The cells were incubated in a suspension of BCECF AM (final concentration 8 μM for 15 min at 22°C). We placed a neutral density (N.D.) filter in front of the excitation light (N.D. 8) to prevent myocyte damage. Fluorescence from BCECF-loaded myocytes was excited and collected in a similar manner to that for fura-2–loaded myocytes, but excitation wavelengths were 440 and 490 nm, and fluorescence at 540 nm was measured. The ratio of fluorescence in-

tensities at 440 and 490 nm excitation was used to estimate pH_i. We measured pH_i during tetanus, and found no detectable changes in cytoplasmic pH during tetanic contraction under our experimental conditions (data not shown).

In vivo calibration of fura-2 and BCECF fluorescence. Values of [Ca²⁺]_i were calculated with the standard equation [15]:

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

In Eq. 1, K_d is the dissociation constant of fura-2 for Ca²⁺, β is the ratio of $F(380)$ at zero [Ca²⁺]_i and saturated [Ca²⁺]_i, $R_{\min}$ is the R value at zero [Ca²⁺]_i, and $R_{\max}$ is the R value at saturated [Ca²⁺]_i. We used the K_d value of 0.24 μM as previously estimated *in vitro* [16, 17]. For *in vivo* estimation of $R_{\min}$, myocytes were treated with 10 μM ionomycin in Ca²⁺-free and glucose-free Tyrode solution containing 4 mM EGTA; the average R value reached steady-state after 30 min, 0.292, and was taken to be $R_{\min}$. The value of $R_{\max}$ was estimated after treatment with 1 μM carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone (FCCP) in Tyrode solution containing 1 mM Ca²⁺; the average R value, $R_{\max}$, was 7.43. The third parameter, β , was estimated with the method proposed by Bakker *et al.* [18]. An instantaneous plot of $F(340)$ vs. $F(380)$ gives the slope m that can be related to β according to Eq. 2:

$$\beta = (mR_{\max} - 1) / (mR_{\min} - 1). \quad (2)$$

Using the average value of m obtained during tetanus in 6 myocytes (not shown), -1.65 , in combination with the values of $R_{\min}$ and $R_{\max}$ (above), β was calculated to be 8.95.

Calibration of the BCECF fluorescence signal was performed by equalizing pH_i and pH_o using the K⁺/H⁺ ionophore nigericin [13, 14]. The cells were superfused with four solutions at different pH (pH range 6.5 to 7.4 adjusted by KOH) containing (in mM): KCl, 140; MgSO₄, 1.2; KH₂PO₄, 1.2; N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid (HEPES), 10; and nigericin, 10 μM . The relationship between BCECF fluorescence ratio and pH was linear in the above pH range. The slope of the curve was 3.14 ratio units per 1 pH unit change in pH. Using the slope of the calibration curve, we estimated the difference in pH.

Measurement of cell length. To monitor changes in cell length simultaneously with fluorescence signals, myocytes were trans-illuminated 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 dis-

tance between the edges was automatically measured with an edge detector (MOPS-SPL-46A001, Hamamatsu Photonics). Signals of fluorescence and cell length were low-pass filtered at 100 Hz and averaged for 1–4 sweeps.

All experiments were performed at room temperature (22 $\pm$ 2°C).

Solutions and chemicals. The composition of the Tyrode solution was (in mM): NaCl, 136.9; KCl, 5.4; MgCl₂, 0.5; NaH₂PO₄, 0.33; HEPES, 5; glucose, 5; and pH was adjusted to 7.40 $\pm$ 0.05 or 7.70 $\pm$ 0.05 with NaOH at 22°C. Ca²⁺ concentration in the Tyrode solution was adjusted by adding a small amount of the CaCl₂ stock solution (1 M). We used bupranolol (1 μM) to inhibit the β -adrenoceptor stimulating effect of phenylephrine throughout the experiments except for the experiment with isoproterenol. All chemicals were of reagent grade: fura-2 AM and BCECF AM (Molecular Probes, Eugene, OR, USA), thapsigargin (Calbiochem, La Jolla, CA, USA), bupranolol (Kaken Pharmaceutical Co., Ltd, Tokyo, Japan), and FCCP, ionomycin, isoproterenol, phenylephrine, prazosin, ethylisopropyl-amiloride (EIPA), and chelerythrine (Sigma Chemical). Thapsigargin and chelerythrine were dissolved in dimethylsulfoxide (DMSO) to make a 5 mM stock solution. EIPA was dissolved in ethanol to make a 5 mM stock solution. The final concentration of DMSO plus ethanol was less than 0.3%, which did not influence either fluorescence signals or cell shortening. The direct effects of isoproterenol, phenylephrine, prazosin, EIPA, and chelerythrine on the fura-2 and BCECF fluorescence signals were tested *in vitro* and were negligible at the concentrations used in the present study.

Statistics. The averaged values were given as mean $\pm$ SEM. Statistical comparisons were carried out using Student's *t*-test with the significance level set at $p < 0.05$.

RESULTS

Effect of isoproterenol on the steady-state relationship between cytoplasmic calcium and cell shortening

Myocytes were treated with thapsigargin (0.2 μM) for 10–15 min to inhibit the Ca²⁺ pump of the sarcoplasmic reticulum [19] and activated by a 10 Hz field stimulation for 10 s delivered every minute to induce tetanic contraction. We measured [Ca²⁺]_i and cell length change (shortening) simultaneously to construct an instantaneous plot of [Ca²⁺]_i and cell length (Ca–L trajectory) for estimation of Ca²⁺ responsiveness. Figure 1 shows typical records of [Ca²⁺]_i (A)

Fig. 1. A and B: $[Ca^{2+}]_i$ and cell shortening (% shortening) during tetanus. A: Changes in $[Ca^{2+}]_i$ during tetanus at 1 mM $[Ca^{2+}]_o$. Solid line, control; broken line, 3 nM isoproterenol. B: Cell shortening during tetanus as shown in A. Solid line, control; broken line, 3 nM isoproterenol. The degree of shortening is expressed as the % change from the resting cell length. C: Instantaneous plots of $[Ca^{2+}]_i$ vs. % shortening of cell length (Ca-L trajectory) from the data shown in A and B. Solid squares, control; open circles, 3 nM isoproterenol.

and cell shortening (B) obtained from the same cell. Each value of $[Ca^{2+}]_i$ was plotted instantaneously against cell shortening to form a phase-plane trajectory (Ca-L trajectory) as we previously reported. At first, we investigated the effect of β -adrenoceptor agonist, isoproterenol (3 nM), on Ca^{2+} responsiveness. As shown in Fig. 1C, isoproterenol shifted the Ca-L trajectory to the right, indicating a decrease in Ca^{2+} responsiveness as reported previously [20]. The results confirmed that the Ca-L trajectory could be used to monitor the Ca^{2+} responsiveness of myofilaments.

Effect of phenylephrine on the steady-state relationship between cytoplasmic calcium and cell shortening

We investigated the effect of α_1 -adrenoceptor agonist, phenylephrine, on Ca^{2+} responsiveness in the presence of bupranolol (1 μ M), which was used to inhibit the effect of phenylephrine on β -adrenoceptor. Phenylephrine slightly but significantly shifted the Ca-L trajectory to the left (indicating an increase in Ca^{2+} responsiveness) in 3 min, and the effect gradually became greater after 15 to 20 min. Therefore, we evaluated the maximal effect of phenylephrine 30 min after drug application. Figure 2A shows the Ca-L trajectory records taken before and after application of phenylephrine (10 μ M) at 1 mM extracellular $[Ca^{2+}]$ ($[Ca^{2+}]_o$). To estimate the extent of the shift of Ca-L trajectory, we measured the $[Ca^{2+}]_i$ which caused 5% shortening from the resting cell length during relaxation phase ($Ca_{5\%}$) and calculated the change in $Ca_{5\%}$ from the control value. Changes in $Ca_{5\%}$ were

Fig. 2. A: Instantaneous plots of $[Ca^{2+}]_i$ vs. % shortening of cell length (Ca-L trajectory) before and after α_1 -adrenoceptor stimulation. Solid squares, control; open circles, 10 μ M phenylephrine (Phe). $[Ca^{2+}]_o$: 1 mM. B: Concentration-dependent effect of phenylephrine on the Ca-L trajectory. Changes in $Ca_{5\%}$ (nM) from the control value obtained from three different concentrations of phenylephrine. Each column represents mean $\pm$ SEM from 5 cells. *: $p < 0.05$ vs. control (paired t -test).

Fig. 3. A: Changes in the Ca-L trajectory induced by an agonist and an antagonist of α_1 -adrenoceptors. Solid squares, control; open circles, 10 μ M phenylephrine (Phe); solid line, 10 μ M phenylephrine plus 1 μ M prazosin. $[Ca^{2+}]_o$: 1 mM. **Inset:** summary of changes in $Ca_{5\%}$ (nM) after application of 10 μ M phenylephrine and 10 μ M phenylephrine plus 1 μ M prazosin. **B: Changes in the Ca-L trajectory induced by phenylephrine and EIPA.** Solid squares, control; open circles, 10 μ M phenylephrine; solid line, 10 μ M phenylephrine plus 5 μ M EIPA. **Inset:** summary of changes in $Ca_{5\%}$ from the control value after application of 10 μ M Phe and 10 μ M phenylephrine plus 5 μ M EIPA. In the insets, each column represents mean $\pm$ SEM from 5 cells. *: $p < 0.05$ vs. control (paired t -test).

38.0 ± 12.0 nM at 1 μ M, 70.6 ± 9.6 nM at 10 μ M, and 74.2 ± 15.8 nM at 100 μ M phenylephrine ($n=5$, Fig. 2B). As shown in Fig. 2B, the effect of phenylephrine on Ca^{2+} responsiveness was almost saturated at 10 μ M, and no significant change was observed between 10 and 100 μ M. Thus, we used 10 μ M phenylephrine in the following experiments.

To test whether or not the shift caused by phenylephrine was mediated via the α_1 -adrenoceptors, we examined the effect of a selective α_1 -adrenoceptor antagonist, prazosin (1 μ M), on the Ca-L trajectory shifted by phenylephrine. Figure 3A shows that 1 μ M prazosin could reverse the shift of the trajectory induced by phenylephrine to the control line. Changes in $Ca_{5\%}$ were 38.6 ± 5.1 nM at 10 μ M phenylephrine and -1.3 ± 7.6 nM at 10 μ M phenylephrine plus 1 μ M

Fig. 4. Effect of PKC-inhibitor on the Ca-L trajectory. Open squares, control; solid squares, 2 μ M chelerythrine; solid line, 2 μ M chelerythrine plus 10 μ M phenylephrine (Phe).

prazosin ($n=5$, Fig. 3A inset). Thus, the shift of Ca-L trajectory induced by phenylephrine is probably mediated through α_1 -adrenoceptors.

Previous reports [3, 4, 21, 22] suggested that phenylephrine activates an Na/H exchanger and increases cytoplasmic pH (pH_i). Therefore, we investigated whether or not the shift of the trajectory induced by phenylephrine could be influenced by inhibiting Na/H exchange. EIPA (a Na/H exchanger inhibitor) alone did not markedly shift the Ca-L trajectory, but significantly shortened it (not shown). Since EIPA decreased the Ca^{2+} signal and cell shortening, the trajectory was shortened without shift. We confirmed that an increase in $[Ca^{2+}]_o$ from 2 to 4 mM recovered the Ca^{2+} signal and cell shortening and extended the Ca-L trajectory to approximately match the control without any shift in the trajectory. The change in $Ca_{5\%}$ was 2.3 ± 1.6 nM at 5 μ M EIPA ($n=3$). Figure 3B shows that 5 μ M EIPA could reverse the shift of the Ca-L trajectory induced by phenylephrine to the control line. Changes in $Ca_{5\%}$ were 65.6 ± 8.1 nM at 10 μ M phenylephrine and 5.3 ± 4.6 nM at 10 μ M phenylephrine plus 5 μ M EIPA ($n=5$, Fig. 3B inset). Thus, the shift in the Ca-L trajectory caused by phenylephrine appears to be somehow related to the activation of Na/H exchange.

It has been reported that phenylephrine activates PKC [4, 22, 23]. We examined the effect of a PKC inhibitor on the Ca-L trajectory. Figure 4 shows the results of an experiment in which phenylephrine (10 μ M) was applied to a cell pretreated with 2 μ M chelerythrine, a PKC-inhibitor. Chelerythrine alone did not shift the Ca-L trajectory. In the presence of chelerythrine, phenylephrine did not alter the Ca-L trajectory. Changes in $Ca_{5\%}$ were 0.1 ± 4.7 nM at 2 μ M chelerythrine and 2.1 ± 6.6 nM at 2 μ M chelerythrine

Fig. 5. Time course of the changes in $Ca_{5\%}$ (A) and pH_i (B) after application of 10 μM phenylephrine (Phe). In A and B, each data point represents mean $\pm$ SEM ($n=6$ for A, $n=11$ for B). *: $p < 0.05$ vs. control (paired t -test).

plus 10 μM phenylephrine ($n=8$).

Effect of phenylephrine on cytoplasmic pH_i

We examined the effect of phenylephrine on pH_i . After BCECF loading, cells were stimulated to produce tetanus, and fluorescence signal were measured at the resting state between tetanic contractions [14]. The value of pH_i was 7.09 ± 0.02 ($n=23$) in the relaxed cells and did not significantly change during tetanus. After the application of 10 μM phenylephrine, pH_i increased in a time-dependent manner (Fig. 5B), while $Ca_{5\%}$ gradually decreased as described above (Fig. 5A), indicating an increase in Ca^{2+} responsiveness. Average pH_i change (ΔpH_i) induced by 10 μM phenylephrine was $+0.12 \pm 0.01$ units in 30 min ($n=11$, the left column in Fig. 7C). We also investigated the effect of prazosin and EIPA on the phenylephrine-induced ΔpH_i . The increase in pH_i by 10 μM phenylephrine was strongly inhibited by 1 μM prazosin ($n=5$, Fig. 6A), and was completely reversed by 5 μM EIPA ($n=5$, Fig. 6B). We also measured the changes in pH_i during chelerythrine treatment. Chelerythrine did not alter pH_i and inhibited the increase in pH_i induced by phenylephrine ($\Delta pH_i = -0.01 \pm 0.02$ with 2 μM chelerythrine; $\Delta pH_i = -0.02 \pm 0.02$ with 2 μM chelery-

Fig. 6. A: Changes in pH_i from the control value induced by 10 μM phenylephrine (Phe, left) and 10 μM phenylephrine plus 1 μM prazosin (right). B: Changes in pH_i from the control value induced by 10 μM phenylephrine (left) and 10 μM phenylephrine plus 5 μM EIPA (right). In A and B, each column represents mean $\pm$ SEM from 5 cells. *: $p < 0.05$ vs. control (paired t -test).

thrine plus 10 μM phenylephrine, $n=7$).

Alkalinization of the cytoplasm could be produced by increasing extracellular pH (pH_o). When pH_o was increased from 7.4 to 7.7, pH_i increased by about 0.1 pH_i units. The average ΔpH_i value ($\Delta pH_i = +0.12 \pm 0.01$, $n=5$) was found to be very similar to that induced by 10 μM phenylephrine (Fig. 7C). The increase in pH_o from 7.4 to 7.7 also caused a leftward shift in the $Ca-L$ trajectory (Fig. 7A) by an extent similar to that induced by 10 μM phenylephrine (Fig. 7B). Changes in $Ca_{5\%}$ from the control were 60.0 ± 5.2 nM at 10 μM phenylephrine ($n=20$) and 69.7 ± 7.3 at $pH_o=7.7$ ($n=8$). These results suggest that the shift in the $Ca-L$ trajectory induced by phenylephrine could be explained by cytoplasmic alkalinization.

DISCUSSION

The present study showed that the mechanism for increasing Ca^{2+} responsiveness by α_1 -adrenoceptor stimulation could be mainly explained by an increase in pH_i due to an increase in Na/H exchange activity through the PKC-mediated pathway. The results are novel because the mechanism for increasing Ca^{2+} responsiveness by α_1 -adrenoceptor stimulation in intact single myocytes was clarified by measuring both the relation between $[Ca^{2+}]_i$ and cell shortening at steady-state in tetanic contraction, not in single-twitch contraction, and pH_i .

Estimation of myofibrillar responsiveness to Ca^{2+} using tetanic contraction in ventricular myocytes. In the present study, we produced

Fig. 7. A: Ca-L trajectory measured at $pH_o=7.4$ (solid squares), and $pH_o=7.7$ (open circles). **B:** Changes in $Ca_{5\%}$ from the control value induced by $10 \mu M$ phenylephrine (Phe, $n=20$) and extracellular alkalosis ($pH_o=7.7$, $n=8$). **C:** Changes in pH_i from the control value induced by $10 \mu M$ phenylephrine (Phe, $n=11$) and extracellular alkalosis ($pH_o=7.7$, $n=5$). In B and C, each column represents mean $\pm$ SEM. n.s.: no significant difference between the two data sets.

tetanic contraction in single myocytes to estimate myofibrillar responsiveness to Ca^{2+} in α_1 -adrenoceptor stimulation. This method enables us to readily estimate a steady-state relation between intracellular Ca^{2+} and cell shortening in intact myocytes that well preserve membrane receptors, second messengers, and signal transduction pathways. Gambassi *et al.* [3] reported that α_1 - and β -adrenoceptor stimulation shifted the re-lengthening phase of the phase-plane loop in twitch shortening to the left or to the right respectively, indicating an alteration in Ca^{2+} responsiveness. The contractile element and intracellular Ca^{2+} are nearly equilibrated in the re-lengthening phase in twitch [7]. However, the quantitative evaluation of the trajectory shift was not performed in their studies. In particular, the late phase of the trajectory shows two

components and the horizontal shift of the trajectory is difficult to evaluate in their study. In addition to these problems, the active cross-bridges tend to detach in the re-lengthening phase of the phase-plane loop.

According to Krueger [8], the re-lengthening phase of unloaded twitch shortening is seriously influenced by the repulsion (restoring) force of the elastic component existing in the cell. In contrast to Gambassi *et al.* [3], the present results showed a very narrow loop of the relationship between $[Ca^{2+}]_i$ and cell shortening, and we could measure Ca^{2+} responsiveness when the contractile elements were almost equilibrated with Ca^{2+} and active cross-bridges were formed. Since we converted the fura-2 ratio to $[Ca^{2+}]_i$, a semi-quantitative change in Ca^{2+} responsiveness, similar to that measured in pCa-tension relation in isometric contraction, was feasible. Thus, the present results are more reliable than those measured using the trajectory of unloaded twitch contraction. As suggested in our previous paper, we used the value in the falling phase of the trajectory because we could not detect a significant difference in the rising and falling phase of the trajectory [9].

Effect of phenylephrine on the steady-state relationship between cytoplasmic calcium and cell shortening.

Gambassi *et al.* [3] previously showed that a positive inotropic effect of an α_1 -adrenoceptor agonist, phenylephrine, is due to an increase in Ca^{2+} responsiveness in rat ventricular myocytes using the trajectory of a single twitch. In the present study, we found that phenylephrine increased myofibrillar responsiveness to Ca^{2+} under our experimental conditions. As shown in Fig. 2B, the effect of phenylephrine on Ca^{2+} responsiveness was almost saturated at $10 \mu M$, in agreement with previous reports [3, 4]. The effect of phenylephrine was completely blocked by prazosin (Fig. 3A), indicating that the effect of phenylephrine was mediated exclusively through the α_1 -adrenergic receptor.

It has been suggested that the effect of phenylephrine is due to the activation of Na/H exchange [22]. We found, in the present study, that EIPA, a selective inhibitor of Na/H exchange [24], reversed the effect of phenylephrine on Ca^{2+} responsiveness (Fig. 3B). This result is compatible with a previous report [3], and indicates that the increase in the activity of Na/H exchange is related to the increase in myofibrillar responsiveness to Ca^{2+} in α_1 -adrenoceptor stimulation. Terzic *et al.* [4] proposed that phosphorylation of the myofilament probably plays a major role in increasing Ca^{2+} responsiveness during α_1 -adrenoceptor stimulation. According to their reports, myocytes were treated with phenylephrine before skinning and then

measured after skinning. They observed a leftward shift in the pCa–tension relation after phenylephrine treatment. The effect of phenylephrine was not significantly influenced by pretreatment with EIPA. The ineffectiveness of EIPA reported by Terzic *et al.* [4] is in contrast to the present results that the leftward shift of the Ca–L trajectory induced by phenylephrine was fully antagonized by EIPA (Fig. 3B). The different results are probably due to the different preparations and procedures, but the details of the mechanisms are not clear. Although they observed an increase in pH_i by +0.1 pH units during α_1 -adrenoceptor stimulation, the contribution of an increase in pH_i to the increase in Ca^{2+} responsiveness is not considered to be a major factor. They did not directly measure the pCa–tension relation when pH in the solution was increased. Therefore, the present result more directly supports the hypothesis that the positive inotropic effect of α_1 -adrenoceptor stimulation is due to an increase in myofibrillar Ca^{2+} responsiveness through an increase in pH_i .

Effect of alkalization on the steady-state relationship between cytoplasmic calcium and cell shortening. The increase in pH_i during α_1 -adrenoceptor stimulation was, on average, $+0.12 \pm 0.01$ pH units ($n=11$), which was similar to the range between +0.08 and +0.10 reported by Terzic *et al.* [4]. Phenylephrine induced a change in Ca^{2+} responsiveness and pH_i with a similar time course (Fig. 5). Treatment with either prazosin or EIPA reversed the phenylephrine-induced effects on both Ca^{2+} responsiveness and pH_i back to the control level (Fig. 6). These results indicate a close correlation between the changes in pH_i and Ca^{2+} responsiveness. We further tested if the small change in cytoplasmic pH (+0.12 pH units) could significantly increase Ca^{2+} responsiveness. When pH_o was increased from 7.4 to 7.7, the average increase in pH_i (+0.12) was comparable to that caused by phenylephrine (Fig. 7C). The Ca–L trajectory was also shifted to the left to a similar extent as that observed during α_1 -adrenoceptor stimulation (Fig. 7B). These results further support the hypothesis that an increase in Ca^{2+} responsiveness can be primarily attributed to an increase in pH_i .

Snabaitis *et al.* [22] reported that α_1 -adrenoceptor stimulation activates Na/H exchange through two independent pathways that involve extracellular signal-regulated kinase (ERK) and PKC. In this study, however, the inhibition of PKC alone almost completely antagonized the phenylephrine-induced effects on Ca^{2+} responsiveness and pH_i . The result suggests a relatively minor role, if any, of the ERK pathway in α_1 -adrenoceptor stimulation regarding the change in

Na/H exchange activity, at least under the present experimental conditions. Therefore, we conclude that an increase in Ca^{2+} responsiveness by α_1 -adrenoceptor stimulation is primarily mediated by the activation of Na/H exchange possibly through a PKC-signaling pathway. However, further studies are needed to clarify the detailed molecular mechanisms for activation of Na/H exchange leading to cytoplasmic alkalization.

Clemént *et al.* [25] suggested that phosphorylation of myosin light chain 2 by PKC together with phosphorylation of troponin-I and troponin-T are involved in the modulation of cardiac contractility. Although the involvement of the contractile element phosphorylation by PKC in the change in Ca^{2+} responsiveness [23, 26] is not clear, our results support the assumption that intracellular alkalization could explain the increase in Ca^{2+} responsiveness by α_1 -adrenoceptor stimulation.

In summary, we conclude that: (1) α_1 -adrenoceptor stimulation increased Ca^{2+} responsiveness; (2) the increase in Ca^{2+} responsiveness can be mainly explained by cytoplasmic alkalization; and (3) the activation of Na/H exchange, possibly through a PKC-mediated pathway, is responsible for alkalization.

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