

Ca²⁺ TRANSIENTS AND CELL SHORTENING IN DIABETIC RAT VENTRICULAR MYOCYTES

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To investigate the role of abnormal Ca²⁺ metabolism in the diminished contractile function of diabetic myocardium, we measured the Ca²⁺ transients and cell contraction of diabetic rat myocytes. Isolated diabetic (8 weeks after 40 mg/kg streptozotocin, i.v.) and normal myocytes were loaded with acetoxymethyl ester of indo-1 (indo-1/AM). Ca²⁺ transients and cell circumferential shortening were measured simultaneously, using high temporal resolution video image analysis. The diastolic base and systolic peak of Ca²⁺ transients were significantly lower in diabetic myocytes than in normal myocytes (peak ratios: 0.49 ± 0.02 vs 0.56 ± 0.01 , $p < 0.05$; base ratios: 0.43 ± 0.01 vs 0.48 ± 0.01 , $p < 0.01$, Mean \pm SE). The cell circumferential shortening of diabetic myocytes was also significantly lower than that of normal myocytes ($2.9 \pm 0.3\%$ vs $5.2 \pm 0.9\%$, $p < 0.05$). Although isoproterenol (10^{-8} and 10^{-7} M) increased the peak of Ca²⁺ transients in both diabetic and normal myocytes, the peak value of Ca²⁺ transients in the diabetic group was significantly lower than that in the normal group. The decreased Ca²⁺ transients may be responsible for the decreased contractile function in diabetic myocardium.

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DIABETES mellitus has been associated with a specific cardiomyopathy in humans and experimental animals¹ which was termed “diabetic cardiomyopathy”. Regan et al^{2,3} have shown that small vessel lesions in diabetic myocardium had little or no relationship to diabetic cardiomyopathy in clinical and experimental data, suggesting that the contractile function of the diabetic myocardium was primarily defective. Although many studies have been performed to reveal cellular and subcellular derangement

in diabetic cardiomyopathy, the precise mechanism is still unknown. Ca²⁺ metabolism in diabetic myocardium reportedly has several abnormalities, including a decreased activity of Ca²⁺ ATPase in sarcoplasmic reticulum⁴ and sarcolemma⁵. It has also been reported that the activity of Na⁺/Ca²⁺ exchange is lower in diabetic myocardium⁶. These reports have suggested the involvement of Ca²⁺ overload in the pathogenesis of diabetic cardiomyopathy. However, we have recently reported⁷ that the intracellular Ca²⁺ concentration ([Ca²⁺]_i) of unstimulated diabetic myocytes was lower than that of normal myocytes using fura-2 (53 ± 3 nM in diabetic myocytes

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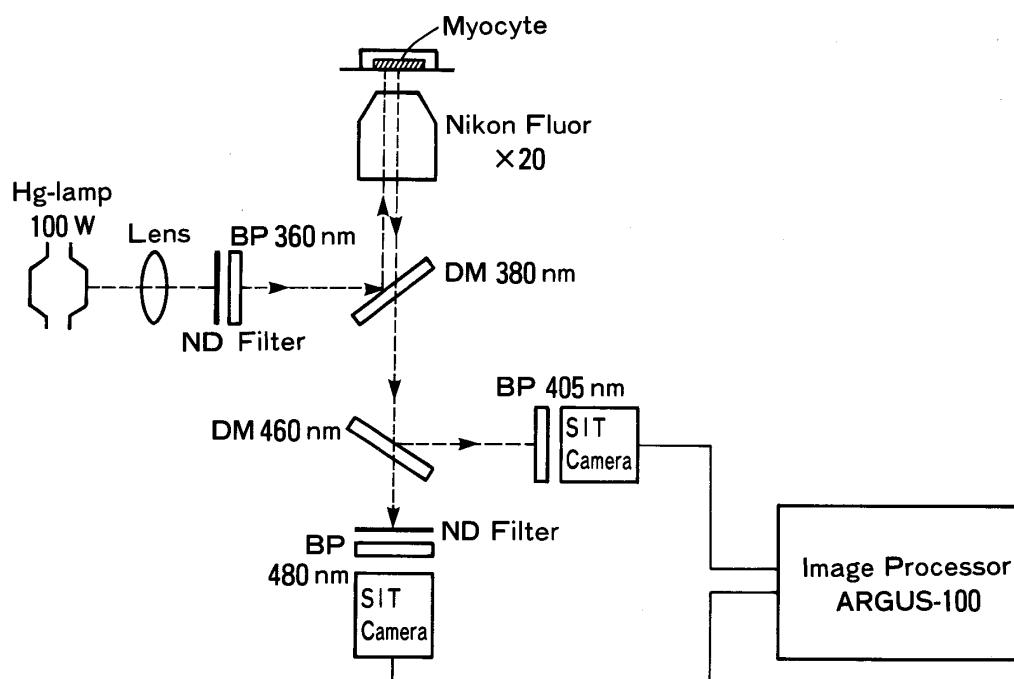


Fig.1. Schematic diagram of epifluorescence optical system. The fluorescence of indo-1 loaded myocyte was imaged using a Nikon TMD inverted microscope (Nikon Fluor 20x), equipped with a 100 W mercury lamp. Emission of indo-1 fluorescence at 405 nm and 480 nm was obtained using a 360 nm bandpass filter for excitation, a 380 nm dichroic mirror and a 460 nm dichroic mirror. All filters had half-bandwidths of 10 nm. Video images were obtained using 2 S.I.T. cameras fed to a computer (ARGUS-100; Hamamatsu Photonics K.K.), and fluorescent ratios were obtained by dividing, pixel by pixel, the 450 nm image by the 480 nm image.

and 75 ± 5 nM in normal myocytes, means \pm SE, $p < 0.01$). Horackova and Murphy have also reported that the Ca^{2+} content is decreased in diabetic rat myocytes using radioisotopes⁸. Ca^{2+} transients of diabetic rat myocytes have not yet been measured directly, although it is widely accepted that the rapid changes of $[\text{Ca}^{2+}]_i$ regulate the contractile function in the myocardium.

In the present study, we measured Ca^{2+} transients of diabetic rat myocytes using time-resolved indo-1 fluorescence microscopy with 2 silicon intensified target (SIT) cameras. Indo-1 is suitable for measuring rapid changes in $[\text{Ca}^{2+}]_i$ without a cell movement artifact, because it measures the 2 wavelengths of emission without changing the excitation wavelength⁹. We measured cell shortening using high temporal resolution video imaging analysis.

METHODS

Induction of diabetes

Male Wistar rats (200–220 g) in the diabetic group received 40 mg/kg streptozotocin in a citrate buffer (0.1 M citric acid and 0.1 M sodium citrate, pH 4.5) intravenously through the tail vein, while age-matched rats in the normal group were treated with a citrate buffer alone. The rats in the 2 groups were maintained for 8 weeks with free access to food and water. Blood glucose was measured every 2 weeks after the injection of streptozotocin using the glucose oxidase method. Diabetic rats with blood glucose levels less than 400 mg/dl were not used. Body weights were determined before sacrifice.

Cell isolation procedure

Ventricular myocytes were isolated from normal and diabetic rats which had been anesthetized with diethyl ether. The heart was excised, cannulated directly onto a retrograde perfusion apparatus (Langendorff column, 60 cm height), subjected to retrograde perfusion through the aorta with a

TABLE I

	Normal (n=5)	Diabetic (n=6)
Body Wt. (g)	399±8	203±10**
Heart Wt. (g)	1.37±0.39	1.13±0.04**
Heart Wt./Body Wt. (mg/g)	3.43±0.09	5.62±0.21**
Blood Glucose (mg/dl)	128±6	497±26**

Table I: General characteristics of normal and diabetic rats used in this study. Values are means±SE. Asterisks indicate significant differences ($p<0.01$) vs normal rats.

solution gassed with 95% O₂–5% CO₂ and maintained at 37 °C. The first perfusate was the Ca²⁺-free modified Krebs' solution (mM); NaCl 113.1, KCl 4.6, MgCl₂ 1.2, NaH₂PO₄ 3.5, NaHCO₃ 21.9, and glucose 10 (pH 7.4) to wash out blood remaining in the heart cavities and coronary arteries. After 3–4 min of initial perfusion, a Ca²⁺-free modified Krebs' solution containing collagenase (50 unit/ml, Yakult, Japan) was added to the column and perfused for 10 min. Finally, the storage solution (mM); KOH 70, l-glutamic acid 50, KCl 40, taurine 20, KH₂PO₄ 20, MgCl₂ 3, glucose 10, HEPES 10, and EGTA 0.5, pH to 7.4 with KOH, was introduced to wash out the residual enzyme solution in the heart. The ventricles were cut into small fragments with iris scissors, and myocytes were dispersed by gentle agitation in the storage solution. Just before the loading of indo-1, the storage solution was exchanged with a modified Krebs' solution containing 0.5 mM CaCl₂. After the isolation procedure, 60–70% of all cells were rod-shaped with clear striae. There was no difference between the percentage of rod-shaped cells in normal and diabetic rats.

Measurement of [Ca²⁺]_i transients

The cells were loaded with 2 μM acetoxymethyl ester of indo-1 (indo-1/AM) for 5 min at 37 °C, and then washed 3 times with modified Krebs' solution which contained 0.5 mM CaCl₂. The cells were placed in an experimental chamber, which was mounted on the stage of a Nikon TMD inverted microscope and a Nikon Fluor 20×, (numerical aperture: 0.75) objective. Rod-shaped myocytes were superfused with modified

Krebs' solution at 30 °C, and electrically stimulated with a frequency of 1 Hz, at twice the threshold amplitude (field stimulation with bipolar Ag-AgCl electrode). The optical system used for the simultaneous measurement of [Ca²⁺]_i and cell circumferential shortening is illustrated in Fig. 1. An indo-1 loaded cell was excited at 360 nm with a 100 W mercury lamp, and the indo-1 emission signal was separated into 405 nm and 480 nm wavelengths. Both images were obtained separately using 2 silicon intensified target (SIT) cameras (C2400-08H, Hamamatsu Photonics K.K., Japan) and stored in a digital imaging processor (ARGUS-100, Hamamatsu Photonics K.K., Japan) at 33 msec intervals. One frame memory consisted of 512×483 pixels. Fluorescent ratios were obtained by dividing, pixel by pixel, the 405 nm image by the 480 nm image after background subtraction in both images. Physical alignment of the 2 images was achieved by superimposing 1 cell image on the other cell image before image acquisition. It was difficult to quantify [Ca²⁺]_i because large amounts of unhydrolyzed indo-1/AM and compartmentation to intracellular organelles were present¹⁰. Therefore, we used the fluorescent ratio of 405/480 nm to represent [Ca²⁺]_i in this study. A higher fluorescent ratio indicated a higher [Ca²⁺]_i.

Measurement of cell shortening

We measured cell circumferential shortening using indo-1 fluorescent images with an ARGUS-100 (Hamamatsu Photonics K.K., Japan). The fluorescent images of the stimulated myocytes were continuously stored in the frame memory at 33 msec intervals. We determined the "threshold" of fluorescence, and pixels which were less than the threshold were excluded from the analysis. Areas which had a fluorescent intensity stronger than the threshold were taken into the ARGUS-100 as a cell image. Thereafter, the ARGUS-100 counted the number of pixels in the outline of the cell image, and used the number of pixels in the vertical and horizontal axes to calculate the cell circumferential length

Statistical analysis

Data for all experiments are presented as means±SE. Differences were analyzed by

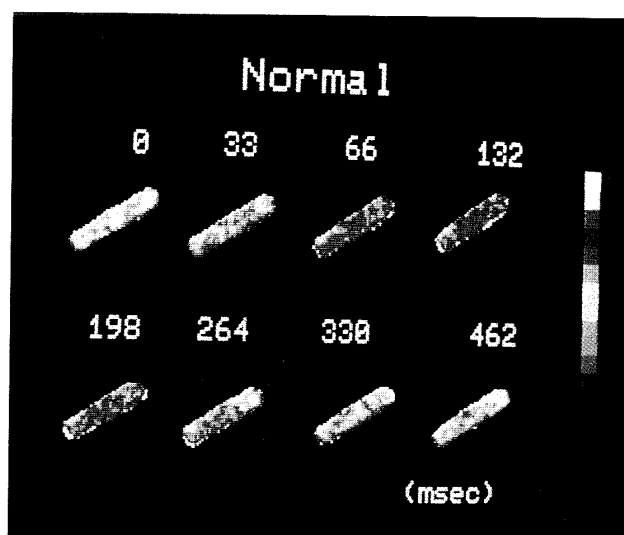


Fig. 2. Images of the fluorescent ratios of an electrically stimulated normal myocyte. Higher colours on the vertical colour-scale on the right indicate that $[Ca^{2+}]_i$ is high.

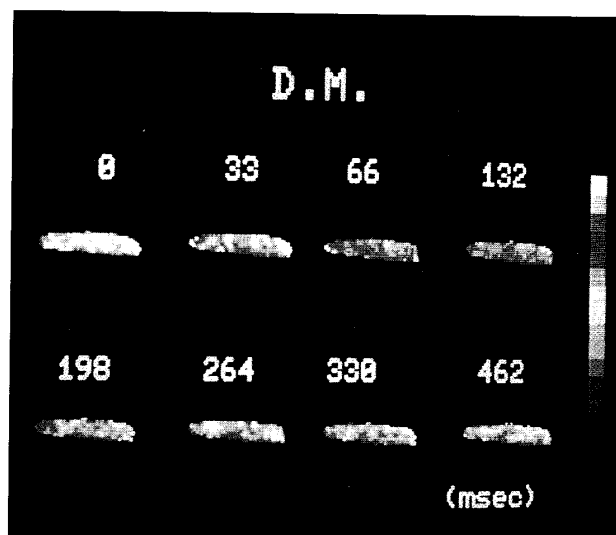


Fig. 3. Images of the fluorescent ratios of an electrically stimulated diabetic myocyte. Higher colours on the vertical colour-scale on the right indicate that $[Ca^{2+}]_i$ is high.

the Student's t-test, and p values less than 0.05 were considered significant.

RESULTS

General characteristics of animals

As shown in Table 1, body weight and heart weight of diabetic rats were significantly smaller than those of age-matched control rats. However, the heart-to-body weight ratio was significantly higher in diabetic rats than in control rats. Blood glucose concentration was significantly higher in diabetic rats. Although ketonuria was seen in some diabetic rats at 1 week after the injection of streptozotocin, ketonuria was not observed in diabetic rats from 2 to 8 weeks after the injection. There was no difference in the cell lengths between diabetic and normal myocytes. (diabetic: $104.5 \pm 1.8 \mu\text{M}$, $n=20$; normal: $103.3 \pm 2.3 \mu\text{M}$, $n=20$).

Ca²⁺ transients and cell contraction

Fig. 2 shows typical images of the fluorescent ratios of an electrically stimulated normal myocyte. The colour indicates the fluorescent ratio (emission at 405/480 nm). The higher colours on the vertical colour-scale on the right indicate a higher fluorescent ratio, i.e., a higher $[Ca^{2+}]_i$. When the myocyte was electrically stimulated, $[Ca^{2+}]_i$ began to increase. The myocyte then started

to contract and $[Ca^{2+}]_i$ increased further. Subsequently, $[Ca^{2+}]_i$ and cell shape returned to the resting level.

Fig. 3 shows typical images of the fluorescent ratios of an electrically stimulated diabetic myocyte. After stimulation, $[Ca^{2+}]_i$ and cell shape show the same pattern as that of the normal myocyte in Fig. 2. However, the systolic peak $[Ca^{2+}]_i$ of the diabetic myocyte was apparently lower than that of the normal myocyte.

Fig. 4 shows the time course of the fluorescent ratio and the circumferential shortening of the myocytes in Fig. 2 and 3 (A: normal myocyte, B: diabetic myocyte). The Ca^{2+} transients reached a peak about 100 msec after the stimulation, while the peak of circumferential shortening appeared 200–300 msec after the stimulation. Both the diastolic level and the systolic peak of Ca^{2+} transients in the diabetic myocyte were lower than those in the normal myocyte.

Table II summarizes the Ca^{2+} transients (diastolic level and systolic peak of fluorescent ratios) and the % of cell circumferential shortening in normal and diabetic myocytes. Both the diastolic level and the systolic peak of Ca^{2+} transients were significantly lower in diabetic myocytes than in normal myocytes. The average value of cell circumferential shortening was also lower in diabetic myocytes. There was no difference between

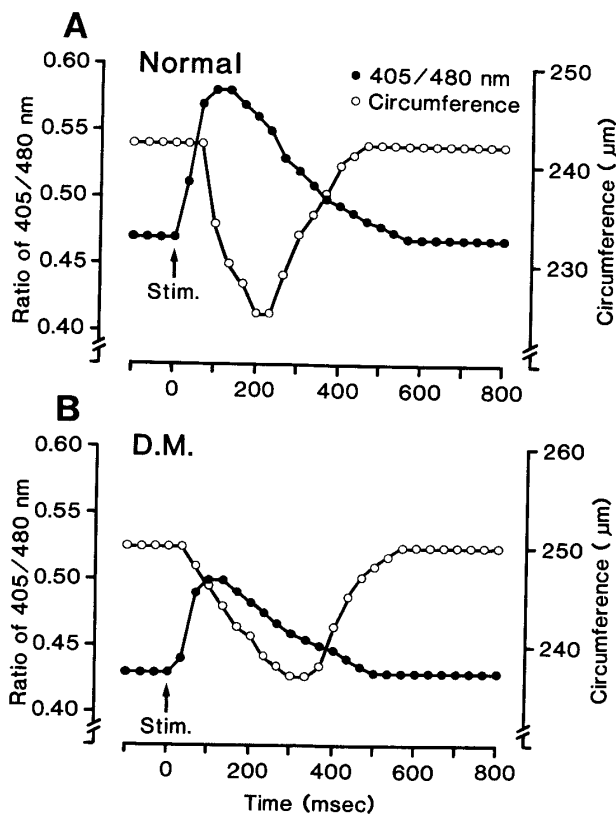


Fig. 4. Typical time course of the fluorescent ratios (405/480 nm) and the cell circumferential shortening of stimulated myocytes. (A) Normal: normal myocyte. (B) D.M.: diabetic myocyte. Stim.: Time at which the myocytes were electrically stimulated.

the time course of either Ca²⁺ transients or cell circumferential shortening (time to peak, total duration and the half decay time) in the 2 groups.

If the activities of cellular esterases in diabetic and normal myocytes differ, the values of [Ca²⁺]_i can not be compared: a low activity of cytosolic esterases could lead to increased indo-1 compartmentation in intracellular organelles, which may, in turn, lead to a lower estimation of [Ca²⁺]_i.¹⁰ Therefore, we compared the calibration value and the residual fluorescence after the addition of 50 μM digitonin between diabetic and normal myocytes. The cells were loaded with indo-1/AM, and then superfused with glucose-free buffer which contained the metabolic poisons carbonyl cyanide m-chlorophenylhydrazone (CCCP: 5.0 μM: an inhibitor of oxidative phosphorylation) and amytal (3.3 mM: an inhibitor of NADH dehydrogenase) for 15–20 min to deplete

TABLE II

A: Ca²⁺ transients

	Normal (n=5)	Diabetic (n=6)
Diastolic level	0.48±0.01	0.43±0.01**
peak	0.56±0.01	0.49±0.02*
Time to peak (msec)	83±9	86±8
Total duration (msec)	416±31	403±24
T _{1/2} (msec)	197±12	183±13

B: Circumferential shortening

	Normal (n=5)	D.M. (n=6)
% of shortening	5.2±0.9	2.9±0.3*
Time to peak (msec)	178±17	205±16
Total duration (msec)	442±42	482±50
T _{1/2} (msec)	165±21	172±16

Table II: Ca²⁺ transients (A) and cell circumferential shortening (B) of normal and diabetic myocytes. Normal: normal myocytes, n=5 from 5 rats. Diabetic: diabetic myocytes, n=6 from 6 rats. T_{1/2}: the time to half decay of Ca²⁺ transients. T_{1/2}: the time to half decay of circumferential length. Values are means±SE. Asterisks indicate significant differences (*: p<0.05 vs normal, **: p<0.01 vs normal).

stores of intracellular ATP. After this procedure, the cells had a “brick-like” appearance and did not contract further upon exposure to Ca²⁺. R_{max} was determined by adding Ca²⁺ ionomycin (10 μM) and CaCl₂ (5 mM). R_{min} was determined by adding Ca²⁺ ionomycin (10 μM) and 10 mM EGTA to the external solution. There was no difference between the calibration values of diabetic and normal myocytes (R_{max}: 2.09±0.02 in diabetic myocytes, n=10, 2.08±0.02 in normal myocytes, n=10; R_{min}: 0.32±0.01 in diabetic myocytes, n=10, 0.33±0.02 in normal myocytes, n=10). There was also no difference in the residual fluorescence of indo-1 after the addition of 50 μM digitonin between the diabetic and normal groups (emission at 405 nm: 42.0±4.5% in diabetic myocytes, n=8, 44.3±2.1% in normal myocytes, n=8; at 480 nm: 46.4±5.2% in diabetic myocytes, n=8, 46.0±3.8% in normal myocytes, n=8). These results show that there is no difference in the indo-1 compartmentation between diabetic and normal myocytes.

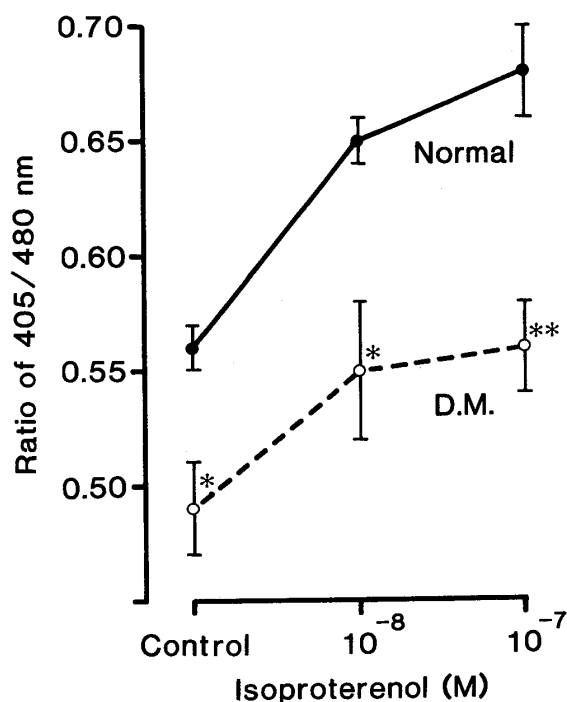


Fig.5. Effects of isoproterenol on the peak fluorescent ratios of Ca^{2+} transients. Normal: normal myocytes ($n=5$). D.M.: diabetic myocytes ($n=6$). Values are means \pm SE. Asterisks indicate significant differences (*: $p<0.05$ vs normal, **: $p<0.01$ vs normal).

Therefore, it is reasonable to compare the indo-1 fluorescence between diabetic and normal myocytes.

Effects of isoproterenol

It has been reported that norepinephrine tends to normalize the peak $[\text{Ca}^{2+}]_i$ in myocytes from hypertrophied failing hearts: the peak level of Ca^{2+} transients is lower in hypertrophied failing hearts than in normal hearts during control perfusion¹¹. Since decreased Ca^{2+} transients in diabetic myocytes might reflect a stronger dependence on neurohormonal factors to maintain contractile performance as in the hypertrophied failing hearts, we investigated the effects of isoproterenol on Ca^{2+} transients. Fig. 5 shows the effects of isoproterenol (10^{-8} and 10^{-7} M) on the systolic peak of Ca^{2+} transients. In both normal and diabetic myocytes, the systolic peaks of fluorescent ratios were significantly enhanced by isoproterenol. However, the peak fluorescent ratios of diabetic myocytes during the perfusion with isoproterenol (at both 10^{-8} and

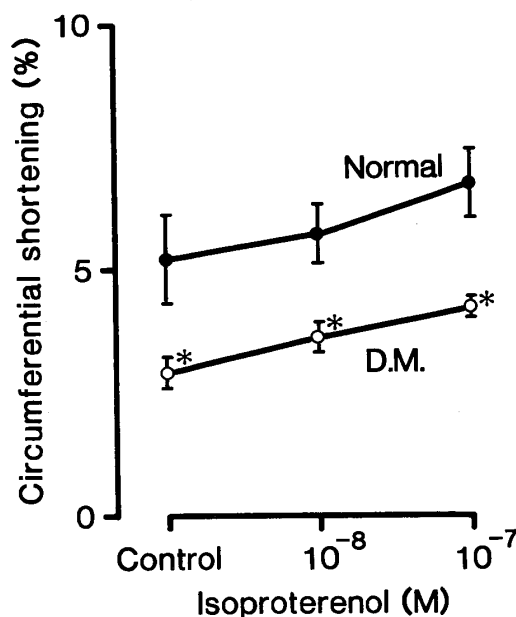


Fig.6. Effects of isoproterenol on cell circumferential shortening. Normal: normal myocytes ($n=5$). D.M.: diabetic myocytes ($n=6$). Values are means \pm SE. Asterisks indicate significant differences (*: $p<0.05$ vs normal).

10^{-7} M) were significantly lower than those of normal myocytes. There was no significant change in the diastolic level of Ca^{2+} transients during the perfusion with isoproterenol (data not shown). Fig. 6 shows the effects of isoproterenol on cell circumferential shortening. Although cell circumferential shortening was significantly increased by isoproterenol (10^{-8} and 10^{-7} M) in both normal and diabetic myocytes, it remained lower in the diabetic myocytes than in the normal myocytes.

DISCUSSION

Clinical and experimental studies have shown that there is depressed ventricular function in diabetic hearts, in the form of a diminished developed tension and a diminished velocity of contraction^{12,13}. However, there are difficulties in interpreting the abnormal function of diabetic myocardium because of coexisting small vessel lesions or hormonal effects. In this study, we showed that the diminished contractile function was independent of hormonal effects or vascular constraints, using isolated ventricular myocytes of diabetic rats. The impairment of force generation in diabetic myocardium

could be due to the derangement of 3 elementary parameters in the excitation-contraction coupling process¹⁴: 1) the pulse of the intracellular free Ca²⁺ concentration that occurs during each cardiac cycle (Ca²⁺ transients), 2) the sensitivity of myofilament to [Ca²⁺]_i, 3) maximal Ca²⁺-activated force. There has been no consistent finding regarding the sensitivity of the myofilament. It has been reported that the lower activity of myosin ATPase and abnormal myosin isozyme distribution could be responsible for the depressed contractile function in diabetic myocardium¹⁵. However, there is no simple correlation between active developed tension and myosin ATPase activity^{16,17}. Murat et al¹⁸ have reported that the Ca²⁺ sensitivity of diabetic skinned myocytes is significantly greater than that of normal myocytes, while the maximal activated tension is similar. Therefore, the abnormal contractile function of diabetic myocardium may be primarily due to the abnormalities of Ca²⁺ transients. However, there have been no previous reports on Ca²⁺ transients in the diabetic heart. We showed that the systolic peak of Ca²⁺ transients of diabetic myocytes was significantly lower than that of normal myocytes. Although we have not investigated the Ca²⁺ sensitivity of contractile protein, it is likely that the diminished peak [Ca²⁺]_i during contraction can account for decreased contractile function in diabetic myocardium. The diastolic level of Ca²⁺ transients was also lower in diabetic myocytes, which is consistent with our previous report using fura-2⁷.

There are several problems with using indo-1/AM to measure Ca²⁺ transients and it is difficult to calculate the absolute [Ca²⁺]_i level because of substantial compartmentation to intracellular organelles¹⁰. However, there was no difference in the calibration values (R_{\min} and R_{\max}) and indo-1 compartmentation between diabetic and normal myocytes. Therefore, it is likely that the relative differences of Ca²⁺ transients can be compared in this study.

In this study, it is not clear why both diastolic level and systolic peak of Ca²⁺ transients were lower in diabetic myocytes. Diabetic myocardium may depend more on neurohormonal factors to maintain contractile performance. Therefore, diabetic myo-

cytes might show lower Ca²⁺ transients than normal myocytes because of the experimental conditions in this study (i.e., free from neurohormonal effects). One of these neurohormonal factors is β -stimulant. Previous reports have shown that the number of β -adrenergic receptors was reduced in diabetic myocardium, and that diabetic myocardium was less sensitive to isoproterenol than normal myocardium^{19,20}. However, Austin and Chess-Williams have recently reported that left atria and ventricular papillary muscles from 3-weeks-diabetic rats were more sensitive to positive inotropic effects of isoproterenol than tissues from normal rats²¹. Therefore, the depressed Ca²⁺ transients of diabetic myocytes in this study may reflect a tight dependence on β -stimulants to maintain Ca²⁺ transients and contractile performance. However, our results showed that isoproterenol did not restore the Ca²⁺ transients of diabetic myocytes to the levels seen in normal myocytes.

Our experiments were performed in the presence of a relatively low extracellular Ca²⁺ concentration ([Ca²⁺]_o: 0.5 mM). We could not increase the [Ca²⁺]_o to the physiological level because of the occurrence of Ca²⁺ waves, probably due to the relatively high concentration of indo-1 (loaded at 2 μ M). The response of Ca²⁺ transients to various [Ca²⁺]_o may differ in normal and diabetic ventricular myocytes.

Many other factors regulate the [Ca²⁺]_i level in myocytes. The activities of Ca²⁺ ATPase and Na⁺/Ca²⁺ exchange in sarcolemma are reportedly depressed in diabetic myocardium, and the involvement of Ca²⁺ overload has been implied in the pathogenesis of diabetic cardiomyopathy. However, our results have shown that the diastolic level of Ca²⁺ transients was lower in diabetic myocytes than in normal myocytes. The activity of Na⁺/H⁺ exchange may participate in the control of [Ca²⁺]_i, since the decreased activity of Na⁺/H⁺ exchange could cause a decrease in intracellular Na⁺ concentration ([Na⁺]_i)²² resulting in a decrease in [Ca²⁺]_i via Na⁺/Ca²⁺ exchange. It has been recently reported that the transient redevelopment of force following the initial decrease in tension during acidotic challenge with NH₄Cl was markedly attenuated in diabetic myocardium. The dimin-

ished force redevelopment may be due to the lower increase of $[Ca^{2+}]_i$ due to the lower activity of the Na^+/H^+ exchange.²³ While the lower diastolic $[Ca^{2+}]_i$ which was observed in the diabetic myocytes may be due to the lower $[Na^+]_i$, there are no reports concerning cytosolic Na^+ concentration in diabetic myocardium.

In this study, the peak level of Ca^{2+} transients of diabetic myocytes was lower than that of normal myocytes. Bergh et al have reported that the net influx of Ca^{2+} was significantly reduced in both acute (4 days) and chronic (8 weeks) diabetic rat myocardium.²⁴ The reduction of Ca^{2+} current may be a mechanism of lower Ca^{2+} influx. However, prolonged action potential duration of 30–40-weeks-diabetic myocardium may be due to enhanced Ca^{2+} current.²⁵ It has been recently reported that postrest contraction and rapid cooling contracture, after variable rest intervals, are significantly depressed in diabetic myocytes, and that the reduction of developed tension in diabetic myocardium was a consequence of diminished Ca^{2+} stores in sarcoplasmic reticulum (S.R.).²⁶ Therefore, it is possible that the diminished S.R. Ca^{2+} stores and Ca^{2+} release during contraction are involved in the diminished peak of Ca^{2+} transients in diabetic myocytes, since it is widely accepted that most of the activator Ca^{2+} is released from the S.R. and that this released Ca^{2+} (from S.R.) produces the peak of Ca^{2+} transients in mammalian myocardium.²⁷

In summary, we have observed lower Ca^{2+} transients and cell shortening in diabetic rat myocytes. The lower Ca^{2+} transients may explain the decreased contractile function of diabetic myocardium. The mechanisms of lower Ca^{2+} transients should be studied further.

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