Intracellular Sodium Concentration in Diabetic Rat Ventricular Myocytes

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SUMMARY
We measured intracellular Na\(^+\) concentration ([Na\(^+\)]\(_i\)) of diabetic rat ventricular myocytes using sodium-binding benzofuran isophthalate (SBFI). We used diabetic rats at 8 weeks after the injection of streptozotocin (45 mg/kg i.v.). The level of [Na\(^+\)]\(_i\) during the control perfusion was significantly lower in diabetic myocytes than that in normal myocytes (9.2 ± 0.4 mM v.s. 12.0 ± 0.3 mM, \(p<0.01\)). After the 40 min perfusion of 1 \(\mu\)M hexamethylene amiloride (HMA), [Na\(^+\)]\(_i\) decreased significantly in both groups. However, there was no difference in [Na\(^+\)]\(_i\) between the two groups after the perfusion of HMA. It was suggested that the lower [Na\(^+\)]\(_i\) in diabetic myocytes could be due to the decreased activity of Na\(^+\)/H\(^+\) exchange system. (Jpn Heart J 36: 647–656, 1995)

Key words: Sodium-binding benzofuran isophthalate (SBFI)
Hexamethylene amiloride Streptozotocin Diabetes mellitus Sodium hydrogen exchange

Diabetic hearts of patients or experimental animals have been shown to be associated with a specific cardiomyopathy, which has been termed "diabetic cardiomyopathy".\(^1,5\) Hearts from diabetic animals exhibit depressed contractile force generation\(^1,2,3\) and a slower rate of relaxation than those from control animals.\(^1,2,3\) We have already reported that the resting intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) was lower in diabetic myocytes than in normal myocytes.\(^4\) We have also reported that the cell shortening as well as the diastolic base and systolic peak of Ca\(^{2+}\) transients were significantly depressed in diabetic myocytes than in normal myocytes.\(^5\) The decrease in [Ca\(^{2+}\)]\(_i\) could explain the altered contractile behavior in diabetic hearts. However, the mechanism of lower [Ca\(^{2+}\)]\(_i\) in diabetic myocytes remains unresolved. Although diminished Ca\(^{2+}\) stores of

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Received for publication May 8, 1995.
Accepted July 19, 1995.

647
sarcoplasmic reticulum (SR) in diabetic myocytes might explain the lower peak of Ca\(^{2+}\) transients\(^{6,7}\), the mechanism of lower resting Ca\(^{2+}\) level is unknown. In cardiac myocytes, Na\(^{+}\) is involved in controlling intracellular Ca\(^{2+}\) and pH via Na\(^{+}/Ca^{2+}\) and Na\(^{+}/H^{+}\) exchangers.\(^{8,9}\) For example, a decrease in intracellular Na\(^{+}\) concentration ([Na\(^{+}\)]\(_i\)) could lead to low [Ca\(^{2+}\)]\(_i\), via Na\(^+\)/Ca\(^{2+}\) exchange. The regulation of [Na\(^{+}\)]\(_i\) is mediated mainly through two membrane transport systems, namely the Na\(^+\)/K\(^+\) pump and Na\(^+\)/H\(^+\) exchange, in unstimulated myocytes.\(^{10}\) Changes in these Na\(^+\) transport pathways have been reported in streptozotocin-induced diabetic hearts. A marked decrease in the activity of the amiloride sensitive Na\(^+\)/H\(^+\) exchange has been shown in hearts from diabetic rats.\(^{11,12}\) This inhibition of Na\(^+\)/H\(^+\) exchange could cause a decrease in [Na\(^{+}\)]\(_i\) in diabetic hearts, since Na\(^+\)/H\(^+\) exchange is one of the major Na\(^+\) entry pathways.\(^{13}\) However, [Na\(^{+}\)]\(_i\) of diabetic myocardium has not been measured in myocyte levels.

Several methods such as ion-selective microelectrodes,\(^{8}\) nuclear magnetic resonance,\(^{14}\) and X-ray microanalysis\(^{15}\) have been applied for the measurement of [Na\(^{-}\)]\(_i\). Both the electrode measurement and X-ray microanalysis are destructive to myocytes, and nuclear magnetic resonance requires large quantities of tissue and long acquisition time. Recently, the measurement of [Na\(^{+}\)]\(_i\) using the fluorescent probes was established and this enabled us to measure [Na\(^{+}\)]\(_i\) in intact myocytes.\(^{16}\)

The purposes of the present study were to measure [Na\(^{+}\)]\(_i\) of myocytes from chronic streptozotocin-induced diabetes, and to determine the possible contribution of Na\(^+\)/H\(^+\) exchange in the regulation of [Na\(^{+}\)]\(_i\) in isolated diabetic cardiac myocytes.

**Methods**

**Animals:** Male Wistar rats of 200–250 g in the diabetic group received 45 mg/kg streptozotocin in citrate buffer (0.1 M citric acid and 0.1 M sodium citrate, pH 4.5) intravenously through the tail vein, while control rats in the age-matched group were treated with citrate buffer alone. The rats in the two groups were allowed free access to food and water, and were kept for 8 weeks. Blood glucose was measured every 2 weeks after the injection of streptozotocin using the glucose oxidase method. Diabetic rats with blood glucose less than 400 mg/dl were not used. Body weights were determined immediately 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 to the bottom of the Langendorff column (60 cm height), and perfused with a solution gassed with 95% O\(_2\)–5% CO\(_2\) and maintained at 37°C.
The first perfusate was the Ca\(^{2+}\)-free Krebs solution (in mM): NaCl 113.1; KCl 4.6; MgCl\(_2\) 1.2; NaH\(_2\)PO\(_4\) 3.4; NaHCO\(_3\) 21.9; glucose 10; equilibrated with 95% O\(_2\)-5% CO\(_2\) (pH 7.4) to wash out blood remaining in the heart cavities and coronary arteries. After 3–4 min of initial perfusion, a Ca\(^{2+}\)-free modified Krebs solution containing collagenase (20 mg/100 ml, Nitta, Japan) was added to the column and perfused for 3 min. Finally the modified Kraftbruhe (KB) solution (in mM): KOH 70; KCl 40; MgCl\(_2\) 3.0; KH\(_2\)PO\(_4\) 20; glutamic acid 50; glucose 10; N-2-hydroxyethylpiperazine-N\(^\prime\)-2-ethanesulfonic acid (HEPES) 10, and ethylene glycol-bis (β-aminoethyl ether)-N\(_2\),N\(_\prime\),N\(_\prime\prime\),N\(_\prime\prime\prime\)-tetraacetic acid (EGTA) 0.5, 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 after the isolation procedure, 20–40% of all cells were rod-shaped with clear striae. There was no difference in the percentage of rod-shaped cells between normal and diabetic rats.

**Measurement of [Na\(^{+}\)]\(i\):** The cells were loaded with the acetoxyethyl ester (AM) of 5 \(\mu\)M SBFI (Molecular Probe Inc.) at room temperature for 30 min. The cells were then washed twice in a modified Krebs solution containing 2.45 mM Ca\(^{2+}\) and were incubated for an additional 30 min at room temperature for complete hydrolysis of the dye. A small portion of SBFI loaded cells placed in a Perspex bath was mounted on the stage of a Nikon TMD inverted microscope. The cells were perfused with a modified Krebs solution at room temperature, and illuminated by a transmitted illuminator or ultraviolet (UV) light, via an epifluorescence illuminator from a 100W xenon lamp equipped with an interference filter which had a half bandwidth of 10 nm. Fluorescences of SBFI loaded cells were imaged using a Nikon fluor (× 20 objective). Images were obtained using a silicon intensified target camera (Hamamatsu Photonics) with the output digitized to a resolution of 512 × 512 pixels by an image processor, ARGUS (Hamamatsu Photonics). The excitation wavelengths were 340 and 380 nm. After passing the filters, the exciting light was reflected by a dichroic mirror (400 nm half-pass wavelength). The fluorescent signal was obtained with an emission wavelength at 520 nm after background subtraction. Images of fluorescence ratios were obtained by dividing, pixel by pixel, the 340 nm image by the 380 nm image. *In vivo* calibration for SBFI fluorescence was conducted in the preliminary group of myocytes according to the methods previously reported.\(^{16}\) Briefly, after the loading of SBFI, cells were superfused with calibration solutions with various Na\(^{+}\) concentrations. The calibration solutions were made from appropriate mixtures of high-Na\(^{+}\) (150 mM) and high-K\(^{+}\) (150 mM) solutions. Gramicidin (10 \(\mu\)M) as an ionophore and 100 \(\mu\)M strophanthidin as an inhibitor of the Na\(^{+}\)/K\(^{+}\) pump were added just before use. The observed values of ratios excited at 340/380 nm at the different Na\(^{+}\) concentration were used to solve the three unknown
parameters, \( R_{\min}, R_{\max}, \) and \( K_d \times \beta \) in the standard equation.

\[
[Na^+]=K_d \times \beta \times \frac{(R-R_{\min})}{(R_{\max}-R)}
\]

where \( R \) is the obtained 340/380 nm excitation ratio, \( R_{\min} \) is the ratio at 0 mM Na\(^+\), \( R_{\max} \) is the ratio at saturating \([Na^+]\), \( K_d \) is the dissociation constant, and \( \beta \) is the ratio of the excitation efficiencies of free to Na\(^+\)-bound SBFI at 380 nm.

Results are expressed as means \( \pm \) SE for the indicated number of myocytes obtained from at least 3 rats. Student’s \( t \) test was used for statistical analyses, and the probability was considered significant at \( p < 0.05 \).

**Results**

**General characteristics of the experimental animals:** Table I shows body weight, heart weight, and plasma glucose concentration in diabetic rats and in

<table>
<thead>
<tr>
<th>Table I. General Features of Control and Diabetic Rats</th>
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<tr>
<td>Control (( n = 16 ))</td>
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<tr>
<td>-------------------------</td>
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<tr>
<td>Body Wt (g)</td>
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<tr>
<td>Heart Wt (g)</td>
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<tr>
<td>Heart Wt/Body Wt (mg/g)</td>
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<td>Blood glucose (mg/dl)</td>
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D.M. = diabetic rats; Body Wt = Body weight; Heart Wt = heart weight.
Values are mean \( \pm \) SE. Asterisks indicate significant difference (\( p < 0.01 \)) v.s. control

**Figure 1.** *In vivo* calibration curves of SBFI-AM loaded cells in diabetic and control myocytes. Calibration was conducted using 10 \( \mu \)M gramicidin as an ionophore. Solid line: the calibration curve of control myocytes. Dotted line: the calibration curve of diabetic myocytes. Fluorescent ratios of 340/380 nm at 5, 10 and 50 mM [Na\(^+\)] were shown in normal myocytes (open circles) and diabetic myocytes (closed squares), respectively.
Table II. Compartmentation of SBFI in Normal and Diabetic Myocytes

<table>
<thead>
<tr>
<th>Excitation</th>
<th>Normal (n = 5)</th>
<th>D.M. (n = 6)</th>
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<tr>
<td>340 nm</td>
<td>44 ± 4%</td>
<td>45 ± 4%</td>
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<tr>
<td>380 nm</td>
<td>48 ± 3%</td>
<td>49 ± 4%</td>
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The percentage of residual fluorescence after the addition of 50 mM digitonin. There was no difference between the two groups. Normal=normal myocytes; D.M.=diabetic myocytes.

Figure 2. The level of [Na⁺]i in diabetic and control myocytes. Normal: normal rat myocytes (n = 100). D.M.: diabetic rat myocytes (n = 88). Bars are means ± SE. There was a statistical difference (p < 0.01) in the values of [Na⁺]i between diabetic and control rats.

Age-matched control rats. Eight weeks after streptozotocin injection, diabetic rats exhibited significantly depressed body and heart weights, and elevated heart-to-body weight ratios. Plasma glucose was significantly increased in diabetic rats. These observations are similar to previous work with streptozotocin-induced diabetic rats in our laboratory.⁴)

Measurement of [Na⁺]i by SBFI: Intracellular distribution of the 340/380 nm fluorescence ratios were uniform as we have previously reported⁶ in both normal and diabetic myocytes. Figure 1 shows the in vivo calibration curves generated in SBFI-loaded normal and diabetic myocytes by the addition of 10 μM gramicidin in 5, 10 and 50 mM Na⁺ solutions. There was no significant difference between the ratios in normal and diabetic myocytes in each concentration. The values of
Figure 3. Effect of HMA on \([Na^+]_i\) in diabetic and normal rat myocytes. One \(\mu M\) hexamethylene amiloride (HMA) was applied for 40 min. Normal: normal rat myocytes \((n = 30)\). D.M.: diabetic rat myocytes \((n = 30)\). Values are means \(\pm SE\). Asterisks show the significant differences (**: \(p < 0.01\) v.s. normal rat myocytes, \(p < 0.05\) and \(p < 0.01\) v.s. control). There was no significant difference in the values of \([Na^+]_i\) after HMA between diabetic and control rats.

R\(_{\text{max}}\) were 7.52 and 7.41, and those of R\(_{\text{min}}\) were 3.96 and 3.91, respectively.

If the percentage of intracellular compartmentation of SBFI was different between normal and diabetic myocytes, the value of \([Na^+]_i\) could not be compared. Table II shows the residual fluorescence after the addition of 50 \(\mu M\) digitonin. There were no differences in residual fluorescences at excitation wavelengths 340 and 380 nm between normal and diabetic groups.

**The level of \([Na^+]_i\) in diabetic myocytes:** Figure 2 shows \([Na^+]_i\) of unstimulated normal and diabetic myocytes during the perfusion of the oxygenated Krebs solution. The value of \([Na^+]_i\) in diabetic myocytes was significantly lower than that of normal myocytes \((9.2 \pm 0.4 \text{ mM}, n = 88; 12.0 \pm 0.3 \text{ mM}, n = 100, \text{ respectively}, \ p < 0.01)\).

**Effect of HMA on \([Na^+]_i\) in diabetic and normal myocytes:** To investigate the possible contribution of Na\(^+\)/H\(^+\) exchange to the lower \([Na^+]_i\) in diabetic myocytes, 1 \(\mu M\) hexamethylene amiloride (HMA; a selective inhibitor of Na\(^+\)/H\(^+\) exchange\(^\text{17}\)) was applied to the solution. After the addition of HMA, \([Na^+]_i\) continued to decrease in both normal and diabetic groups, and the steady state \([Na^+]_i\) was obtained at 30 min after the addition of HMA (data not shown). Figure 3 shows the values of \([Na^+]_i\) before and after 40 min perfusion of HMA. \([Na^+]_i\) decreased significantly in both normal and diabetic groups (from 10.9 \(\pm\) 0.6 mM to 6.7 \(\pm\) 0.6 mM, \(n = 30, p < 0.01\); from 8.5 \(\pm\) 0.6 mM to 7.0 \(\pm\) 0.4 mM, \(n = 30, p < 0.05\), respectively). Although \([Na^+]_i\) in diabetic myocytes was
significantly lower than that of normal myocytes before the application of HMA, there was no difference in [Na\(^+\)]\(_i\) between the two groups after the addition of HMA.

**DISCUSSION**

**Measurement of [Na\(^+\)]\(_i\) by the use of SBFI in diabetic myocytes:** We have already reported that the measurement of [Na\(^+\)]\(_i\) in isolated myocytes by the use of fluorescent indicator SBFI was a non-destructive and fast responding method.\(^{16}\) In this study, we applied this method to measure [Na\(^+\)]\(_i\) in diabetic myocytes. There are, however, several problems which should be considered. [1] Compartmentation of SBFI: In this study, about ~50% of fluorescence was retained within the AM loaded cells after the lysis with 50 µM digitonin, suggesting the presence of compartmentation of SBFI in subcellular organelles, presumably mitochondria and SR. The percentage of residual fluorescence after digitonin was similar to that reported by Donoso et al.\(^{18}\) [2] Incomplete hydrolysis of SBFI/AM: Myocytes were loaded with SBFI/AM for 30 min, a relatively short time, and incubated for an additional 30 min at room temperature to gain hydrolyzed fluorescent dye forms. *In vivo* calibration was then done for the measurement of [Na\(^+\)]\(_i\). [3] Photobleaching of SBFI fluorescence: Illumination from the xenon lamp was controlled as low as possible by ND filters, reducing the bleaching of SBFI fluorescence to less than 10% in our experiment. The differences in intracellular environment such as the activities of esterase or the viscosity of cytosol could be the cause of different fluorescent ratios of SBFI. However, there were no differences in the calibration curve (Figure 1) or residual fluorescence after the addition of digitonin between control and diabetic myocytes (Table II). It is, therefore, likely that the relative difference of [Na\(^+\)]\(_i\) between diabetic and control myocytes could be correct.

**[Na\(^+\)]\(_i\) in diabetic myocytes:** This is the first study that measured [Na\(^+\)]\(_i\) in diabetic myocytes, and we showed that [Na\(^+\)]\(_i\) in diabetic myocytes was lower than that of normal myocytes. Conflicting results have been reported by other investigators. Warley demonstrated increased Na\(^+\) concentration in diabetic myocytes by using X-ray microanalysis.\(^{15}\) Kjeldsen also reported increased [Na\(^+\)]\(_i\) in streptozotocin-induced diabetic rat ventricular muscles using flame photometry.\(^{19}\) In these experiments, however, ionic concentrations were measured not from living myocytes or muscles but from frozen or homogenized samples. In unstimulated cardiac myocytes, there are two major mechanisms responsible for the regulation of [Na\(^+\)]\(_i\), namely Na\(^+\)/H\(^+\) exchange and the Na\(^+\)/K\(^+\) pump. We have already reported that [Na\(^+\)]\(_i\) was determined by the balance between these Na\(^+\) pathways.\(^{10}\) It has been reported that the activity of the Na\(^+\)/K\(^+\) pump is
decreased in diabetic myocardium\textsuperscript{20} and that the increase in [Na\textsuperscript{+}]i of the diabetic heart has been explained mainly by this decreased activity of the Na\textsuperscript{+}/K\textsuperscript{+} pump.\textsuperscript{15,19} However, the effect of Na\textsuperscript{+}/H\textsuperscript{+} exchange on [Na\textsuperscript{+}]i has not been studied in diabetic myocytes. Thus, it is possible that if the Na\textsuperscript{+} influx via Na\textsuperscript{+}/H\textsuperscript{+} exchange was significantly inhibited, the level of [Na\textsuperscript{+}]i could be low in diabetic myocytes, even when Na\textsuperscript{+} extrusion via the Na\textsuperscript{+}/K\textsuperscript{+} pump was decreased.

**Decreased activity of Na\textsuperscript{+}/H\textsuperscript{+} exchange in diabetic myocytes:** Intracellular pH and [Na\textsuperscript{+}]i in the heart are regulated by the sarcolemmal Na\textsuperscript{+}/H\textsuperscript{+} exchange. We have already reported that HMA, a specific inhibitor of Na\textsuperscript{+}/H\textsuperscript{+} exchange, decreased [Na\textsuperscript{+}]i of guinea pig myocytes, showing that Na\textsuperscript{+} influx via Na\textsuperscript{+}/H\textsuperscript{+} exchange is active in the control unstimulated myocytes.\textsuperscript{10} In this study, after the addition of 1 \mu M HMA, [Na\textsuperscript{+}]i decreased in both control and diabetic rat myocytes. This observation is consistent with a previous report that has demonstrated a decrease in Na\textsuperscript{+} contents after the inhibition of Na\textsuperscript{+}/H\textsuperscript{+} exchange with amiloride.\textsuperscript{13} Furthermore, there was no difference in [Na\textsuperscript{+}]i after 40 min perfusion of HMA between the two groups. These results suggest that Na\textsuperscript{+}/H\textsuperscript{+} exchange plays an important role in the regulation of [Na\textsuperscript{+}]i in both groups, and that the lower [Na\textsuperscript{+}]i in diabetic myocytes could be due to the depressed activity of the Na\textsuperscript{+}/H\textsuperscript{+} exchange system. The decrease in the activity of Na\textsuperscript{+}/H\textsuperscript{+} exchange has been also shown in hearts from streptozotocin-induced diabetic rat by using ion-sensitive microelectrodes\textsuperscript{10} or biochemical assay.\textsuperscript{11}

It is possible that decreased Na\textsuperscript{+}/H\textsuperscript{+} exchange activity could lead to intracellular acidosis in diabetic myocytes. However, previous studies have demonstrated that the value of pH\textsubscript{i} in diabetic myocytes was not different from control myocytes using a fluorescent indicator\textsuperscript{4} or ion-sensitive microelectrode.\textsuperscript{12} This could be due to the complicated mechanisms regulating intracellular pH\textsubscript{i} such as Na\textsuperscript{+}/H\textsuperscript{+} exchange, Na\textsuperscript{+}/HCO\textsubscript{3}\textsuperscript{-} symport, Cl\textsuperscript{-}/HCO\textsubscript{3}\textsuperscript{-} exchange, intracellular H\textsuperscript{+} buffering, and carbonic anhydrase in cardiac myocytes.\textsuperscript{21}

We have already reported that the low [Ca\textsuperscript{2+}]i and the depressed Ca\textsuperscript{2+} transients could be the reason for the depressed contractility of diabetic myocardium.\textsuperscript{4,5} It has been reported that the decrease in tension in acidosis induced by NH\textsubscript{4}\textsubscript{Cl} withdrawal was followed by the transient recovery of tension and that this transient positive inotropy was markedly attenuated in diabetic rat papillary muscles.\textsuperscript{22} It has been also reported that transient force recovery was suppressed in the presence of amiloride, suggesting the contribution of Na\textsuperscript{+}/H\textsuperscript{+} exchange for the force development.\textsuperscript{22} The lower [Na\textsuperscript{+}]i due to depressed activity of Na\textsuperscript{+}/H\textsuperscript{+} exchange in diabetic myocardium would lead to decreased [Ca\textsuperscript{2+}]i via Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange, which could depress contractile force. Thus, there may be a direct link between the activity of the Na\textsuperscript{+}/H\textsuperscript{+} exchange and cardiac contractility.

Diabetic hearts have been reported to be more resistant to ischemia/
reperfusion injury resulting from cytosolic Ca\(^{2+}\) overload.\(^{23}\) Since Na\(^{+}/H^{+}\) exchange is one of the major Na\(^{+}\) entry pathways during ischemia/reperfusion,\(^{24}\) the decreased Na\(^{+}/H^{+}\) exchange activity would inhibit the increase in [Na\(^{+}\)]\(_i\), and the subsequent increase in [Ca\(^{2+}\)]\(_i\) via Na\(^{+}/Ca^{2+}\) exchange. Therefore, the lower activity of Na\(^{+}/H^{+}\) exchange could prevent Ca\(^{2+}\) overload during ischemia/reperfusion in diabetic myocytes. Further studies are required to clarify the regulation of [Na\(^{+}\)]\(_i\) and the relation between [Na\(^{+}\)]\(_i\) and [Ca\(^{2+}\)]\(_i\) in diabetic myocytes.

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