Stimulus interval-dependent differences in Ca$^{2+}$ transients and contractile responses of diabetic rat cardiomyocytes

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Abstract

Objective: The aim of this study was to gain further insights into the consequences of insulin-dependent diabetes mellitus on cardiomyocyte calcium handling. Methods: The effects of steady state and transient changes in stimulus frequency on the intracellular Ca$^{2+}$ transient and cell shortening were examined in left ventricular cardiomyocytes isolated from the hearts of control and streptozotocin-induced diabetic rats. Results: During steady state stimulation diabetic rat cardiomyocytes displayed a slower decay of the Ca$^{2+}$ transient and longer times for maximum cell shortening and re-lengthening. At 1.5 mM extracellular [Ca$^{2+}$], increasing stimulus frequency over the range 0.2–1.0 Hz led to an increase in resting and peak [Ca$^{2+}$], as well as the amplitude of the transient in both the control and diabetic groups. At frequencies greater than 0.4 Hz the amplitude of the transient was significantly depressed in diabetic rat cells and this was not normalized by increasing extracellular [Ca$^{2+}$] to 2.5 mM. Recovery of sarcoplasmic reticulum (SR) Ca$^{2+}$ release was measured from the time course of restitution of the intracellular Ca$^{2+}$ transient. In both control and diabetic rat cardiomyocytes recovery of the transient occurred in two phases. In diabetic rat myocytes, the initial rapid phase of restitution at intervals <1 s was markedly slowed. The fraction of Ca$^{2+}$ recirculating between the SR and the cytosol was estimated from the decline in amplitude of transients following post-rest potentiation. There was no difference in this fraction between control and diabetic rat cells either at 1.5 or 2.5 mM extracellular [Ca$^{2+}$]. Conclusion: The blunted frequency response of diabetic rat cardiomyocytes at frequencies greater than 0.4 Hz is consistent with reduced SR Ca$^{2+}$ uptake leading to reduced SR Ca$^{2+}$ content and subsequent release. At stimulus intervals greater than 1 Hz this is likely to be exacerbated by slower recovery of SR Ca$^{2+}$ release. Despite the evidence for depressed SR Ca$^{2+}$ uptake, the relative amount of Ca$^{2+}$ recirculating within diabetic rat cardiomyocytes remains unaltered. This is most likely due to an accompanying reduction in Ca$^{2+}$ efflux from the cell due either to depressed Na$^+$/Ca$^{2+}$ exchanger activity, or an elevation in intracellular Na$^+$ levels.

Keywords: Diabetes; Calcium (cellular); Myocytes

1. Introduction

Insulin-dependent diabetes mellitus has several adverse consequences on the heart and vascular system. Epidemiological studies reveal a higher incidence of heart failure in diabetics independent of risk factors such as coronary artery disease and hypertension compared to the non-diabetic population [1]. Clinical indices of heart function in diabetics show a decrease in stroke volume, a reduced ejection fraction in response to exercise (contractile reserve) and impaired left ventricular relaxation [1]. In vitro studies with cardiac tissue obtained from animals where diabetes is chemically induced reveal a reduced capacity to develop force or pressure as well as slower rates of force or pressure development and relaxation [2,3]. These altered contractile properties of the diabetic heart have been linked to disturbances in myocyte Ca$^{2+}$ regulation. Recent studies reveal a slower rate of decay of the Ca$^{2+}$ transient in single cardiomyocytes isolated from diabetic rat hearts [4–7]. Whether differences in resting and peak Ca$^{2+}$ levels also occur is more controversial [8–10]. Biochemical studies demonstrating reduced Ca$^{2+}$ transport by purified

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sarcoplasmic reticulum (SR) and sarcolemmal membrane fractions further support the hypothesis that the capacity of diabetic cardiomyocytes to regulate intracellular Ca\(^{2+}\) levels is impaired [11–14].

Variations in the pattern or rhythm of stimulation are known to influence the strength of contraction of the heart. The steady state contraction–frequency relationship is an intrinsic property of cardiac muscle that reflects the time-dependency of the mechanisms that make Ca\(^{2+}\) available to the myofilaments on a beat-to-beat basis. Two manifestations of the contraction–frequency relationship under non-steady state conditions are mechanical restitution and post-rest potentiation. Mechanical restitution describes the recovery of contractile force as a function of the interval between contractions, while post-rest potentiation describes the increase in contractile strength that occurs when stimulation is recommenced following a prolonged pause. The decline of force following post-rest potentiation has in turn been used to estimate the fraction of Ca\(^{2+}\) that is sequestered by the SR, the ‘recirculation fraction’ [15]. Variations of force during steady state or transient changes in stimulus frequency result from parallel changes in intracellular Ca\(^{2+}\) levels, and consequently steady state contraction–frequency relationship, mechanical restitution and post-rest potentiation have been widely employed as tools for investigating myocardial Ca\(^{2+}\) handling.

Under normal conditions most mammalian species exhibit a positive contraction–frequency relationship. In many models of severe cardiac hypertrophy and failure however this relationship is severely blunted or even reversed [16–18] and appears to arise from defects in intracellular Ca\(^{2+}\) regulation. Mechanical restitution and post-rest potentiation are also sensitive indicators of myocardial Ca\(^{2+}\) handling. Mechanical restitution is slowed in failing human and canine hearts [19,20] while the recirculation fraction (RF) is reduced in hypothyroid rabbit and failing human heart [21,22].

Despite well-documented changes in electrophysiological and SR Ca\(^{2+}\) handling properties, there are reports that frequency-dependent changes in peak force or shortening of diabetic cardiac muscle is normal [4,6,23]. This, however, may not be indicative of the true response of the diabetic heart as even preparations from non-diabetic animals exhibited a negative contraction or [Ca\(^{2+}\)]–frequency relationship. In this situation it is likely that the SR is already fully loaded with Ca\(^{2+}\) and may be unable to respond to interventions that would normally be expected to increase SR Ca\(^{2+}\) stores [24,25]. Therefore, one objective of the present study was to evaluate the effects of stimulus frequency on cardiomyocytes which display a positive contraction or [Ca\(^{2+}\)]–frequency relationship. In addition, there is at present no information on whether diabetes-induced derangements in cellular Ca\(^{2+}\) handling alter mechanical restitution or the recirculation fraction. Since they provide important insights into cardiomyocyte Ca\(^{2+}\) handling pathways, a further objective of this study was to investigate the consequences of diabetes on these two processes. To address these objectives we have measured changes in intracellular Ca\(^{2+}\) concentration in cardiomyocytes isolated from insulin-dependent diabetic rat hearts in response to both steady-state and transient changes in stimulus frequency. Preliminary accounts of this work have been presented [26].

2. Methods

2.1. Induction of diabetes mellitus

Insulin-dependent diabetes mellitus was induced in male Wistar rats weighing 275–325 g through a single tail vein injection of 60 mg/kg of streptozotocin (Sigma) dissolved in 0.1 M citrate buffer (pH 4.5). A control group of rats received only citrate buffer. All animals were allowed free access to food and water, and were kept for 7 weeks following injection. The development of diabetes was verified by overt physical signs including lack of weight gain, polydipsia and polyuria, and was confirmed by blood glucose measurements made at the time the animals were killed. All procedures for the care and handling of animals were in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, and were approved by the Monash University Animal Ethics Committee.

2.2. Isolation of cardiac myocytes

Rats were deeply anaesthetized with chloroform and were killed by decapitation. The heart was quickly excised, cleared of adhering tissue and perfused by the Langendorff method at 37°C with a modified Krebs–Henseleit solution. The composition of the solution was (mM): 118.0 NaCl, 4.8 KCl, 1.2 MgSO\(_4\), 1.2 KH\(_2\)PO\(_4\), 10.0 HEPES, 10.0 glucose and 1.5 CaCl\(_2\). The pH of the solution was adjusted to 7.4 and it was oxygenated by bubbling with 100% O\(_2\). When the heart rate was regular the perfusate was changed to a nominally Ca\(^{2+}\)-free Krebs–Henseleit solution. Hearts were perfused with the Ca\(^{2+}\)-free solution for 5 min once contractions had ceased. Collagenase (Type II, Worthington, UK) was then added to the perfusate to a final concentration of 150 I.U./ml and perfusion was continued for a further 20 min. The heart was then removed from the perfusion apparatus and the left and right ventricles were separated. The left ventricle was cut into small pieces and was incubated with gentle shaking with 10 ml of the collagenase medium for 5 min. The collagenase solution was decanted and the dispersed cells were collected by centrifugation at 30 g for 1–2 min. Incubation of the tissue with collagenase and collection of cells was repeated five to six times. The collected cells were resuspended in 3–5 ml of the Krebs–Henseleit...
solution containing 2.5% bovine serum albumin and were gently agitated until used.

2.3. Measurement of intracellular $\text{Ca}^{2+}$

Intracellular $\text{Ca}^{2+}$ was measured using fura-2. Cells were loaded with fura-2 by incubating them at 22°C for 10 min with 5 μM fura-2/AM (Molecular Probes) dissolved in the same solution used to suspend the cells. An aliquot of the fura-2-loaded cell suspension was placed in a plexiglas chamber and the cells were allowed to settle and adhere to the coverslip which formed its base. The chamber was attached to the stage of a Nikon Diaphot 300 inverted microscope.

Fluorescence was measured using a Cairn spectrophotometer (Cairn Research, Kent, UK) as previously described [7]. A xenon lamp in conjunction with a rotating filter wheel provided excitation light at 340 and 380 nm. The emitted fluorescence at 510 nm was measured using a photomultiplier. A rectangular diaphragm immediately before the photomultiplier was adjusted to restrict recording to a single cell within the field of view and a shutter preserved light from reaching the cells during non-recording periods. The filter wheel was rotated at 100 Hz, and for each rotation values of $F_{340}$ and $F_{380}$ were measured and their ratio ($F_{340}$/$F_{380}$) which represents the cytosolic free $\text{Ca}^{2+}$ concentration was determined. The sampling rate was therefore 100 Hz. Fluorescence values were not corrected for cell auto-fluorescence because in preliminary experiments the fluorescence recorded from unloaded cells was near the limits of detection. At the end of each experiment fluorescence ratios were calibrated as previously described [7]. There were no significant differences in the values of $B$, $R_{\max}$, or $R_{\min}$ [27] between control and diabetic groups. The values for control rat cells ($n=13$) were 7.6±0.5, 13.5±0.7 and 0.8±0.02, respectively, while the corresponding values for diabetic rat cells ($n=10$) were 6.4±0.5, 14.1±0.5 and 0.8±0.03. Since accurate calibration of fluorescence ratios knowledge of a number of additional parameters (extent of non-cytosolic distribution of fura-2, extent of incomplete hydrolysis, etc.) [28] which might be variable from cell to cell, results are expressed as $F_{340}$/$F_{380}$ rather than as $\text{Ca}^{2+}$ concentrations. If it is assumed that these and the $K_{d}$ for Ca$^{2+}$ and fura-2 remain unchanged in diabetic rat cells, then it is likely that differences in $F_{340}$/$F_{380}$ values reported here reflect real differences in intracellular $\text{Ca}^{2+}$.

2.4. Measurement of myocyte contractility

Measurements of cell length during contraction were made using a line scan camera and digital imaging technique similar to that previously described [29]. Briefly, a 1×512 element photodiode array was positioned along the longitudinal axis of the cell by means of a rectangular mask located in the optical path of the microscope phototube. Cell length was scanned at intervals of 1.088 ms throughout the contraction cycle with scanning synchronized to commence with stimulus delivery. The camera output of successive scans was digitized and displayed as an image. Calibration at 1 pixel=0.36 μm was determined by scanning a stage graticule. Cell boundary positions were determined for each scan. For each contraction recorded this information was analyzed to derive values for the following contraction parameters. Maximum shortening (%S) was expressed as a percentage of initial resting cell length ($L_{o}$). The time to the onset of cell shortening ($T_{o}$) was measured as the time for cell length to decrease to 0.005×$L_{o}$. The time to peak shortening ($T_{p}$) was measured as the time of first occurrence of %S, while the duration of the twitch ($T_{v}$) was the time required for cell length to return to 0.005×$L_{o}$. The maximum rate of shortening (MRS) and maximum rate of re-lengthening (MRL) were also derived. All time parameters were referred to the commencement of scanning synchronized with the delivery of the stimulus to the cell. Contraction parameters were averaged for the final five stimuli at each frequency.

2.5. Experimental protocols

All experimental protocols were conducted at 25°C, and unless otherwise indicated at 1.5 or 2.5 mM extracellular [Ca$^{2+}$]. Cells were selected for recording only if they displayed normal, rod-shaped morphology with clear and regular sarcomere spacing, were quiescent in the absence of stimulation, and responded to electrical stimulation with synchronous contractions. Electrical stimulation was achieved with suprathreshold square wave pulses of 1–4 ms duration delivered by platinum field electrodes placed in close proximity to the cell.

The effects of stimulus frequency on intracellular $\text{Ca}^{2+}$ were measured during steady state stimulation at 0.2, 0.3, 0.4, 0.5, 0.75 and 1.0 Hz. Cells were allowed 3 min following each change in frequency for their contractile response to stabilize. Measurements of cell contractility were compared at 0.3 and 1.0 Hz for cells not loaded with fura-2.

Restitution of the $\text{Ca}^{2+}$ transient was measured as previously described [22]. Cells were stimulated at 0.2 Hz, and when a steady state had been achieved, a single test stimulus was delivered at either 0.5, 0.75, 1.0, 1.25, 1.5, 1.75, 2, 3, 4 or 5 s following a steady state contraction. The order of extrasystolic stimuli was randomly varied. Cells were allowed 3 min between each experimental run to re-establish a steady state.

To measure the recirculation fraction cells were stimulated at 1.0 Hz until the $\text{Ca}^{2+}$ transient amplitude had attained steady state. Stimulation was then stopped for 30 s and recommenced at 0.2, 0.3, 0.4 or 0.5 Hz. The recirculation fraction was determined as the slope of the linear
relationship that exists between the amplitude of post-rest contractions \( n \) and \( n - 1 \) (see Results). A 5-min period was allowed between experimental runs for cells to return to a steady state at 1.0 Hz.

The lower temperature employed for these experiments helped minimise loss of fura-2 from the cells and ensured that cell viability could be maintained over long periods when cells were subjected to multiple protocols. Usually each of the protocols (frequency, restitution and recirculation fraction) was undertaken on each cell in a randomized order. Cell viability was assessed by the absence of spontaneous contractile activity and was determined at the completion of each protocol. Any cell judged to be compromised was discarded, and any remaining protocols were completed with a new cell. By this means the \([Ca^{2+}]_c\)–frequency relationship, restitution and the recirculation fraction were studied in each heart.

2.6. Statistical analysis

Data for all experiments are expressed as means±standard error of the mean (SEM). Except where otherwise stated \( n \) refers to the number of cells, and in each case data from one cell for each animal were included in any given comparison. The unpaired Student’s \( t \)-test was used to compare the effects of diabetes on body weight, blood glucose concentration, myocyte dimensions, the various parameters of cell contractility and the \( Ca^{2+} \) transient. Where appropriate the Bonferroni correction for multiple comparisons was applied. The effects of stimulus frequency on the \( Ca^{2+} \) transient in control and diabetic rat myocytes were compared separately using one-factor analysis of variance with repeated measures. Only cells where measurements were obtained at every frequency were included in this analysis. In all cases \( P \) values <0.05 were considered significant.

3. Results

3.1. Characteristics of experimental preparations

A total of 13 control and 13 STZ-treated animals were used in the present study. The body weight of rats assigned to the control group increased from \( 322±12 \) to \( 501±10 \) g over the 7-week treatment period. In contrast, STZ-treated rats failed to gain any weight. The mean body weights of this group were \( 281±21 \) g at the beginning and \( 284±16 \) g at week seven. Blood glucose levels of the STZ-treated rats were markedly elevated confirming the development of insulin-dependent diabetes. The values were \( 6.1±0.4 \) mM prior to treatment and \( 19.3±0.5 \) mM at week 7. In preliminary experiments we have found that treatment of rats with STZ leads to hyperglycemia within 7 days and this is maintained over the treatment period. The corresponding blood glucose values for the control group were \( 4.5±0.4 \) and \( 5.0±0.4 \) mM, respectively.

Cell dimensions (length and width) were compared in ten cells from each of five control and five diabetic animals (50 cells in total for each group). There was no difference in cell length between the two groups. Mean values were \( 127±5 \) \( \mu \)m for the control group and \( 122±3 \) \( \mu \)m for the diabetic group (\( P>0.05 \)). Cell width in the control group was \( 33±1 \) \( \mu \)m compared to \( 27±1 \) \( \mu \)m for the diabetic group (\( P<0.05 \)).

3.2. Effects of stimulus frequency

Fig. 1 shows representative recordings of \( Ca^{2+} \) transients obtained from a control and a diabetic rat heart myocyte during steady state stimulation at 0.2 Hz and 1.0 Hz. Increasing stimulus frequency consistently resulted in an increase in resting and peak \( F_{340/380} \) levels as well as the amplitude of the transient in both groups. The effects of frequency at 1.5 mM extracellular \([Ca^{2+}]_c\) are summa-
rized in Fig. 2A–C. The $[\text{Ca}^{2+}]_{i}$–frequency relationship was positive for myocytes from both control and diabetic animals. Over the frequency range 0.2–1.0 Hz both control and diabetic rat heart cells exhibited a statistically significant, frequency-dependent increase in resting $F_{340/380}$, peak $F_{340/380}$ and amplitude of the transient.

Values of resting $F_{340/380}$ were slightly, though not significantly, higher in diabetic rat cardiomyocytes while peak $F_{340/380}$ values, particularly at frequencies $>0.4$ Hz, tended to be lower. As a consequence, there were significant differences between the control and diabetic rat cells in the amplitude of the transient at the higher frequencies. The same differences in peak $F_{340/380}$ and the amplitude of the transient between control and diabetic groups were evident when the values were expressed relative to those measured at 0.2 Hz (data not shown). Relative changes in resting $F_{340/380}$ were identical in the two groups.

It has been suggested that increasing SR $\text{Ca}^{2+}$ content can partly or fully reverse the depressed contractility observed in diabetic rat cardiac muscle [23]. Therefore to investigate whether the $[\text{Ca}^{2+}]_{i}$–frequency relationship could be restored to normal in diabetic rat myocytes, the effects of frequency were also investigated at 2.5 mM extracellular $[\text{Ca}^{2+}]$. These experiments were performed on a different population of cells to those used for the 1.5 mM $\text{Ca}^{2+}$ experiments, and the results are summarized in Fig. 2D–F. In control rat myocytes the positive $[\text{Ca}^{2+}]_{i}$–frequency relationship was maintained with a significant frequency-related increase in each $\text{Ca}^{2+}$ transient parameter. In diabetic rat myocytes there was also a significant frequency-dependent increase in resting and peak $F_{340/380}$, but in this case the increase in peak $F_{340/380}$ was due solely to the increase in resting $F_{340/380}$ and there was no change in $\text{Ca}^{2+}$ transient amplitude. In both groups the effects of frequency were less pronounced at 2.5 mM extracellular $[\text{Ca}^{2+}]$ compared to 1.5 mM extracellular $[\text{Ca}^{2+}]$. For example, in control and diabetic rat cells the amplitude of the transient increased by 80.2 and 46.1%, respectively, at 1.0 Hz compared to 0.2 Hz when the extracellular $[\text{Ca}^{2+}]$ was 1.5 mM. The corresponding increases for the control and diabetic groups at 2.5 mM extracellular $[\text{Ca}^{2+}]$ were 26.8 and 0%. Differences in $\text{Ca}^{2+}$ transient amplitude between control and diabetic groups were still evident at 2.5 mM extracellular $[\text{Ca}^{2+}]$ and were significantly different at frequencies greater than 0.4 Hz. The differences between control and diabetic groups were also evident when values were expressed relative to those measured at 0.2 Hz (data not shown).

### 3.3. Time course of contraction and the $\text{Ca}^{2+}$ transient

In cells from diabetic animals the decline of the $\text{Ca}^{2+}$ transient was slower as was cell shortening and relaxation. This is illustrated in Fig. 3A and B which show examples of transients and twitches recorded during stimulation at 0.3 Hz. Fig. 3C–F shows comparative kinetic analyses of the transient and cell length during contraction at two

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**Fig. 2.** Effects of stimulus frequency on resting and peak $F_{340/380}$ and the amplitude of the transient at 1.5 mM extracellular $[\text{Ca}^{2+}]$ (A–C) and 2.5 mM extracellular $[\text{Ca}^{2+}]$ (D–F). Results are from $n=6$ control and $n=5$ diabetic rat myocytes at 1.5 mM $[\text{Ca}^{2+}]$ and from $n=8$ control and $n=7$ diabetic cells at 2.5 mM $[\text{Ca}^{2+}]$. *, Significantly different compared to control rat myocytes at the same frequency.
different frequencies. Several parameters were used to evaluate the contractile event. These were the time from
the stimulus to the onset of cell shortening ($T_o$), the time to
peak shortening ($T_M$), the duration of the twitch ($T_F$) and
the MRS and MRL.

The effects of frequency were similar for cells from
control and diabetic animals. An increase in frequency
increased the rate of decay of the transient. It also reduced
the time required for maximum cell shortening to occur as
well as the time required for re-lengthening. Consequently
the duration of the Ca$^{2+}$ transient and the twitch were
shorter at higher frequencies.

The rate of decay of the Ca$^{2+}$ transient was compared in
control and diabetic groups at frequencies of 0.3 and 0.5
Hz by fitting a single exponential function to its declining
phase. As shown in Fig. 3C the time constant of the fitted
function was significantly longer in diabetic compared to
control cells. Contractile parameters were compared at 0.3
and 1.0 Hz in cells not loaded with fura-2. All measures of
the time course of the twitch were prolonged in diabetic rat
heart cells. Fig. 3D–F shows that values for $t_o$, $T_M$ and $T_F$
were all significantly longer in the diabetic group. The
MRS and the MRL also tended to be slower in the diabetic
group. Values for MRS at 0.3 Hz in control ($n=9$) and
diabetic ($n=9$) groups were $3.1 \pm 0.2$ $L_o$/s and $2.4 \pm 0.1$
$L_o$/s, respectively ($P<0.05$). At 1 Hz the corresponding
values were $2.8 \pm 0.2$ $L_o$/s and $2.4 \pm 0.1$ $L_o$/s ($P>0.05$).
Values for MRL at 0.3 Hz in control ($n=9$) and diabetic
($n=9$) groups were $2.6 \pm 0.2$ $L_o$/s and $2.4 \pm 0.1$ $L_o$/s,
respectively ($P>0.05$). The corresponding values at 1 Hz
were $2.3 \pm 0.1$ $L_o$/s and $2.1 \pm 0.1$ $L_o$/s ($P>0.05$). We
observed no effect of diabetes on the maximum extent of
cell shortening at either 0.3 or 1 Hz. For example, at 1.0
Hz, cell shortening expressed as a percentage of resting
cell length was $9.8 \pm 0.7$ and $10.3 \pm 0.9\%$, respectively, in
control ($n=13$) and diabetic rat cells ($n=12$).
3.4. Restitution of the intracellular Ca\(^{2+}\) transient

To investigate whether slower recovery of SR Ca\(^{2+}\) release between contractions might contribute to the reduced peak and amplitude of the Ca\(^{2+}\) transient of diabetic rat myocytes, particularly at higher frequencies, we measured the rate of restitution of the intracellular Ca\(^{2+}\) transient.

The method used in these experiments is similar to that which has been previously used to measure mechanical restitution of cardiac muscle, and is illustrated using examples of original recordings in Fig. 4A–D. Cells were stimulated at 0.2 Hz, and when a steady state had been reached a single test stimulus was delivered at a variable interval following a steady state contraction. The interval between the steady state contraction and the test stimulus was varied over the range 500 ms to 5 s. As shown in Fig. 4A–D the amplitude of the extrasystolic Ca\(^{2+}\) transient is less than the steady state amplitude at short extrasystolic intervals, and it increases towards the steady state amplitude as the extrasystolic interval is lengthened.

The mean data from these experiments conducted at 1.5 mM extracellular [Ca\(^{2+}\)] are summarized in Fig. 4E. In control rat myocytes recovery of the transient occurs rapidly and reaches 90% of the steady state amplitude within the first 1.5 s. It is followed by a slower recovery back to steady-state values. In diabetic rat myocytes recovery of the Ca\(^{2+}\) transient is slowed at extrasystolic intervals <1 s, but is normal at longer intervals. Restitution curves were constructed by fitting exponential functions to the data. In the control group a double exponential function provided the best fit. In the diabetic group a double exponential function also provided the best fit to the data, however the two rate constants were nearly identical indicating that the data could equally as well be described by a single exponential function. In order to compare the initial rapid phase of restitution, the parameters from the double exponential fit have been used. In control cells the early phase of the restitution process occurred with a time constant of 285 ms compared to 756 ms in diabetic cells. Nearly identical results were obtained when restitution of the transient was measured at 2.5 mM extracellular [Ca\(^{2+}\)] (data not shown). The time constant of the initial rapid phase of restitution was 302 ms in the control group and 725 ms in the diabetic group. The results clearly show that at stimulus intervals <1 s, recovery of the SR Ca\(^{2+}\) release process is slowed in diabetic rat cells.

3.5. Recirculation fraction of intracellular Ca\(^{2+}\)

Calcium uptake by the SR loads the SR with Ca\(^{2+}\) in between contractions, while Na\(^+\)/Ca\(^{2+}\) exchange moves Ca\(^{2+}\) across the sarcolemma and out of the cell. A change in the rate of SR Ca\(^{2+}\) uptake or Na\(^+\)/Ca\(^{2+}\) exchange might therefore alter the balance between the amount of Ca\(^{2+}\) recirculating within the cell and the amount extruded out of the cell. The next series of experiments was designed to investigate whether these fractions are altered in diabetic rat myocytes.

Fig. 5A shows a recording from a control rat myocyte
Fig. 5. Example of the protocol used to determine the recirculation fraction. (A) Ca\(^{2+}\) transients recorded from a control rat cell stimulated at 1.0 Hz. This was followed by a 30-s rest and stimulation was recommenced at 0.2 Hz. (B) Relationship between the amplitude of post-rest transient \(n\) and \(n-1\) for this cell. The different symbols represent data points obtained at different post-rest stimulus frequencies. The RF for this cell was 0.80 \((r^2 = 0.815)\). Extracellular \([Ca^{2+}]\) was 1.5 mM.

any difference between control and diabetic rat cells. At 2.5 mM extracellular \([Ca^{2+}]\) the RF was 0.95 \((r = 0.995)\) in control rat cells, 0.95 \((r = 0.995)\) in diabetic rat cells, and 0.97 \((r = 0.996)\) for pooled control and diabetic data. These results indicate that the fraction of \(Ca^{2+}\) recirculating within the cell is unchanged by diabetes.

4. Discussion

4.1. Effects of diabetes on the \([Ca^{2+}]_i\)–frequency relationship

Under conditions of steady state stimulation the amplitude of the \(Ca^{2+}\) transient was lower in diabetic rat cardiomyocytes, particularly at frequencies greater than 0.5 Hz. Calcium transients from diabetic rat cells also declined more slowly. All measured indices of cell shortening and re-lengthening were slowed in diabetic rat myocytes resulting in an increase in twitch duration. These results are generally consistent with previous studies employing ventricular myocytes isolated from insulin-dependent diabetic rat hearts [4–6]. The slower maximum rate of shortening and longer time to maximum shortening might reflect the \(V_i\) to \(V_i\) isomyosin shift that occurs in the diabetic heart, while the longer time required for cells to re-lengthen most probably reflects the slower clearance of cytosolic \(Ca^{2+}\). The high time resolution of the method employed for measurements of cell contractility also allowed accurate determination of cardiomyocyte excitation–contraction coupling latency \((T_o)\). The first observable occurrence of shortening presumably reflects the externally measurable outcome of ongoing shortening against an internal load. The delayed onset of shortening in diabetic rat cardiomyocytes may be partly due to an increase in cellular stiffness, a decrease in myofilament...
Ca\(^{2+}\) sensitivity [31], decreased SR Ca\(^{2+}\) release (see below) or some combination of these factors. At physiological concentrations of extracellular [Ca\(^{2+}\)] we observed no depression of the maximum extent of cell shortening in the diabetic rat cells. This is consistent with the observation that peak \(F_{340/380}\) is also normal at frequencies <0.5 Hz. It is however surprising at higher frequencies where there was a trend to lower peak \(F_{340/380}\) levels and the reduced myofilament sensitivity to Ca\(^{2+}\) that is reported to occur in this model of diabetes [31]. It is possible that the slower clearance of cytosolic Ca\(^{2+}\) allows a longer time for Ca\(^{2+}\) to equilibrate with the myofilaments so that shortening continues for longer.

A major finding in the present study is that the steady state [Ca\(^{2+}\)],–frequency relationship is depressed in diabetic rat cardiomyocytes. There were clear differences between control and diabetic groups in peak \(F_{340/380}\) values and the amplitude of the transient irrespective of whether the data was expressed in absolute terms or expressed relative to a single frequency. In this respect our results contrast with recent studies which indicate that variations in frequency produce essentially a normal relative contractile or [Ca\(^{2+}\)], response in diabetic rat cardiomyocytes [5,6]. The discrepancy may reflect the characteristics of the myocyte preparations employed. Under our experimental conditions and at physiological concentrations of extracellular Ca\(^{2+}\), both control and diabetic rat myocytes responded to an increase in stimulus frequency with an increase in resting and peak [Ca\(^{2+}\)], levels, an increase in the amplitude of the transient and abbreviation of its duration. These responses, which are representative of a positive contraction–frequency relationship, may at first appear uncharacteristic of rat cardiac muscle where the relationship is usually considered to be negative. In fact rat cardiac muscle can show either a positive, negative or flat response to increases in stimulus frequency, and even myocytes isolated from the same heart can show the three distinct patterns [24,32]. The increased amplitude of the transient reflects increased SR Ca\(^{2+}\) content and release, and arises from an increase in time-averaged Ca\(^{2+}\) entry through the L-type Ca\(^{2+}\) channels as well as enhanced Ca\(^{2+}\) entry through Na\(^{+}\)/Ca\(^{2+}\) exchange, secondary to an increase in intracellular [Na\(^{+}\)] [24,33]. In previous studies both control and diabetic rat cardiomyocytes generally displayed a pronounced inverse contraction or [Ca\(^{2+}\)],–frequency relationship [5,6]. In such cells it is likely that SR Ca\(^{2+}\) stores are saturated even at low rates of stimulation and are therefore unable to load any more Ca\(^{2+}\) [24,25]. We would point out that in other respects the myocytes from which recordings were made in the present study displayed Ca\(^{2+}\) responses that are considered typical for rat cardiac muscle e.g. they show post-rest potentiation and a negative staircase following a period of rest.

The depressed [Ca\(^{2+}\)],–frequency relationship in diabetic rat myocytes indicates disturbances in the mechanisms that determine the availability of intracellular Ca\(^{2+}\). In rat cardiac muscle Ca\(^{2+}\) release from the SR is the principle source of Ca\(^{2+}\) for myofilament activation, and the observed decline in the amplitude of the transient in diabetic rat cells compared to control rat cells indicates that SR Ca\(^{2+}\) release is decreased. Frequency-dependent potentiation of SR Ca\(^{2+}\) release reflects the balance between those mechanisms that increase and those that
limit SR Ca$^{2+}$ content and release. It is enhanced by increased sarcolemmal Ca$^{2+}$ entry per unit time as described above. It is limited by the rate-dependent abbreviation of the action potential which limits the time and magnitude of trigger Ca$^{2+}$ influx, and the rate of Ca$^{2+}$ cycling by the SR, which depends in part on the activity of the SR Ca$^{2+}$-pump and the recovery from inactivation of the SR Ca$^{2+}$ release channels. The depressed [Ca$^{2+}$]$_r$–frequency relationship in diabetic rat cardiomyocytes might, therefore, arise through changes in one or more of the above mechanisms. Action potential duration is increased in this model of insulin-dependent diabetes and would be expected to favor, not limit, SR Ca$^{2+}$ loading and release [34]. It is therefore likely that changes in SR function are of greater importance in limiting the response of diabetic cells to increases in stimulus frequency.

There is now considerable evidence that Ca$^{2+}$ accumulation by the SR is depressed in diabetic hearts. The activity of the SR Ca$^{2+}$-pump in diabetic cardiac muscle is reduced [12], which would be expected to slow relaxation and reduce SR Ca$^{2+}$ content. The observation that the amplitudes of rapid-cooling contractures and post-rest contractions are reduced in diabetic cardiac muscle provides direct evidence for diminished SR Ca$^{2+}$ stores [4,5,23]. If the blunted response to stimulus frequency simply reflected reduced SR Ca$^{2+}$ stores in the diabetic rat cells, then it might be expected that the relationship would be normalized if SR Ca$^{2+}$ stores could be restored to similar levels to control rat cells. This was investigated by elevating the extracellular Ca$^{2+}$ concentration to 2.5 mM, which for rat cardiac muscle represents a condition where SR Ca$^{2+}$ stores are nearly saturated [35]. Under these conditions the positive [Ca$^{2+}$]$_r$–frequency relationship was still evident in control rat myocytes although the magnitude of the effects were greatly attenuated. In diabetic rat cells Ca$^{2+}$ transient amplitude became insensitive to frequency, indicating that in these cells the capacity of the SR to further accumulate and store Ca$^{2+}$ may be limited. Differences between control and diabetic groups although somewhat reduced were still evident, suggesting that the reduced SR Ca$^{2+}$ release in diabetic cells may be more complex than simply a normal SR containing less Ca$^{2+}$.

4.2. Restitution of the Ca$^{2+}$ transient

It is known that following one contraction some time is required before another stimulus will produce a contraction of equal strength. This property of cardiac muscle is termed restitution and it reflects the time-dependent recovery of SR Ca$^{2+}$ release [36]. It is possible, therefore, that the blunted frequency response of diabetic cells might be due to slower restitution of SR Ca$^{2+}$ release.

Restitution of the Ca$^{2+}$ transient was determined by interposing extra stimuli during periods of steady state stimulation. In diabetic rat myocytes recovery of the Ca$^{2+}$ transient was slowed at extrasystolic intervals less than 1 s but was normal at longer intervals. The question arises as to which cellular mechanisms may limit SR Ca$^{2+}$ release at short stimulus intervals. The initial rapid phase of mechanical restitution (covering intervals 500–1000 ms) reflects primarily SR function, as it is sensitive to interventions which modify the ability of the SR to store and release Ca$^{2+}$ [36,37]. The time course of restitution of the Ca$^{2+}$ transient will depend on the time required for the Ca$^{2+}$ current ($I_{Ca}$) to recover from inactivation, the rate of sequestration of Ca$^{2+}$ back into the SR, as well as the time required for the SR Ca$^{2+}$ release channels to recover from inactivation. Of these mechanisms recovery of the SR Ca$^{2+}$ release channel is slowest and likely to make the largest contribution to the overall rate of recovery of the transient [15]. Normally, SR Ca$^{2+}$ stores as judged by caffeine or rapid cooling contractures require ≈200–500 ms to restitute completely [38,39]. Recovery of $I_{Ca}$ requires ≈200–1000 ms [40,41] while recovery of the SR Ca$^{2+}$ release channels requires ≈700–1000 ms [15,39]. A slowing of any of these processes would be expected to significantly influence restitution at short extrasystolic intervals. Apart from slower SR Ca$^{2+}$ uptake, there is no direct experimental data regarding restitution of either $I_{Ca}$ or the SR Ca$^{2+}$ release channel in this model of diabetes.

In the genetically derived WBN/Kob rat model of non-insulin-dependent diabetes, which shows many electrophysiological similarities to the STZ model, Tsuchida et al. [42] reported that recovery from inactivation of $I_{Ca}$ is normal. If a similar situation exists in the STZ-treated rat, then it is unlikely that restitution of $I_{Ca}$ is responsible for the slower recovery of Ca$^{2+}$ release. There is even less information regarding the function of the SR Ca$^{2+}$ release channels other than the study of Yu et al. [4] who reported no difference in affinity, but a reduced number of $^{3}$H-ryanodine binding sites in homogenates from diabetic hearts.

Our results indicate that SR Ca$^{2+}$ uptake and release is essentially complete in diabetic rat cells by 1 s and would not therefore be expected to limit SR Ca$^{2+}$ release at stimulus frequencies less than 1 Hz. Therefore we would suggest that at frequencies less than 0.5 Hz where SR Ca$^{2+}$ cycling is complete, there is likely to be sufficient time between contractions for the SR to accumulate released Ca$^{2+}$ and differences between control and diabetic myocytes will be slight. As frequency is increased to 1 Hz and the time available for SR Ca$^{2+}$ uptake is shortened, reduced SR Ca$^{2+}$-ATPase activity in diabetic rat cardiomyocytes leads to reduced SR Ca$^{2+}$ content and its subsequent release. At stimulus frequencies above 1 Hz, SR Ca$^{2+}$ cycling is incomplete and would further exacerbate the capacity of diabetic rat cells to release Ca$^{2+}$.

The other notable difference between control and diabetic rat cells was the change in shape of the restitution curve. Restitution of the Ca$^{2+}$ transient in control cells (and presumably of force), was best approximated by a biexponential function. In diabetic rat cells on the other
hand the process could equally as well be described by a
single exponential function. In cardiac muscle which
shows a well-developed SR and is mostly dependent on SR
Ca$^{2+}$ release for activation (e.g. rat ventricle, atrial tissue)
mechanical restitution is a biexponential process, whereas
in cardiac muscle less dependent on SR Ca$^{2+}$ release (e.g.
rabbit and guinea pig ventricle), restitution is best de-
scribed by a single exponential process [43]. Thus it is
tempting to speculate that this difference between the
control and diabetic groups might reflect a reduced depen-
dence on SR Ca$^{2+}$ release and a greater dependence on transarcolemmal Ca$^{2+}$ entry by diabetic rat car-
diomyocytes.

4.3. Recirculation of intracellular Ca$^{2+}$

Two processes are important in removing Ca$^{2+}$ from the
cytosol on a beat-to-beat basis. The SR takes up most of
the internal Ca$^{2+}$ which is then retained within the cell,
while the Na$^{+}$/Ca$^{2+}$ exchanger extrudes the remaining
Ca$^{2+}$ out of the cell. A change in SR Ca$^{2+}$ uptake or
Na$^{+}$/Ca$^{2+}$ exchanger activity would therefore be expected
to alter the fraction of Ca$^{2+}$ cycling between the cytosol
and the SR.

The fraction of cytosolic Ca$^{2+}$ sequestered by the SR
has been termed the ‘recirculation fraction’, and is usually
estimated from the decay of force when contractility is
transiently enhanced following either post-extrasystolic or
post-rest potentiation [30,44]. The RF is derived from the
slope of the line when the force of one potentiated 2
transiently enhanced following either post-extrasystolic or
post-rest potentiation [30,44]. In the present study we found
that stimulation following a 30-s rest period produced near
maximum potentiation of the amplitude of the first post-
rest transient. Variations of the steady-state post-rest
stimulus frequency then allowed the amplitude of the
transients to decline at different rates to different levels,
thereby providing sufficient data points to allow the RF to
be determined. As shown in Figs. 5 and 6 there is a strong
linear relationship between the amplitude of successive
post-rest transients confirming the validity of this ap-
proach.

Values of the RF in control and diabetic myocytes were
0.90 and 0.88, respectively, when the extracellular [Ca$^{2+}$]
was 1.5 mM. These values are in good agreement with a
value of 0.84 found by Ravens et al. [45] who analyzed the
decay of twitch shortening, and a value of 0.92 determined
by Basani et al. [46]. Elevation of extracellular [Ca$^{2+}$] to
2.5 mM increased the RF in both control and diabetic
groups to 0.95. The increase in RF under these conditions
most likely arises from a reduced driving force for Ca$^{2+}$
efflux through Na$^{+}$/Ca$^{2+}$ exchange, resulting in enhanced
fractional SR Ca$^{2+}$ accumulation.

In view of the evidence supporting reduced SR Ca$^{2+}$-
ATPase activity in diabetic cardiac muscle it was our
expectation that the RF would be reduced. The simplest
and most plausible explanation for the finding that it is not,
is that there must be a corresponding decrease in the
amount of Ca$^{2+}$ lost from the cell such that the RF remains
unchanged. There are two possible mechanisms through
which this might occur. One involves a decrease in activity
of the Na$^{+}$/Ca$^{2+}$ exchanger which is the major route for
Ca$^{2+}$ loss from the cell. Makino et al. [11] have shown
that Na$^{+}$/Ca$^{2+}$ exchange activity is reduced in sarcolem-
mal vesicles isolated from insulin-dependent diabetic rat
hearts, while Schaffer et al. [47] have shown reduced
Na$^{+}$/Ca$^{2+}$ exchange activity in non-insulin dependent
diabetic rat hearts. The second involves an increase in the
intracellular Na$^{+}$ concentration. The activity of the sar-
colemmal Na$^{+}$–K$^{+}$ ATPase and the Na$^{+}$–H$^{+}$ exchanger,
both of which regulate levels of intracellular Na$^{+}$ are
depressed in diabetic hearts and lead to higher intracellular
Na$^{+}$ levels [48–50]. This in turn will reduce Ca$^{2+}$
efflux through Na$^{+}$/Ca$^{2+}$ exchange and favor Ca$^{2+}$
uptake by the SR.

5. Conclusions

The results of this study reveal clear differences in the
intracellular Ca$^{2+}$ transient and Ca$^{2+}$ handling properties
of left ventricular cardiomyocytes from insulin-dependent
diabetic rat hearts. The blunted [Ca$^{2+}$]-frequency rela-
tionship in diabetic rat myocytes at frequencies <1 Hz
most likely reflects reduced SR Ca$^{2+}$ uptake and release,
and would be further compounded by slower recovery of
SR Ca$^{2+}$ release at frequencies >1 Hz. However in the
diabetic rat heart the relative amount of Ca$^{2+}$ cycling
between the cytosol and the SR remains unchanged. Since
there is normally a tight coupling between intracellular
[Ca$^{2+}$] and contractile force, we would predict a similar
depression of the force–frequency relationship in diabetic
hearts. Given that increases in heart rate represent an
important mechanism for increasing cardiac output, our
results indicate that the effectiveness of this mechanism
may be limited in the diabetic heart.

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