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Mechanisms underlying the frequency dependence of contraction and $[Ca^{2+}]_i$ transients in mouse ventricular myocytes

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In most mammalian species force of contraction of cardiac muscle increases with increasing rate of stimulation, i.e. a positive force–frequency relationship. In single mouse ventricular cells, both positive and negative relationships have been described and little is known about the underlying mechanisms. We studied enzymatically isolated single ventricular mouse myocytes, at 30 °C. During field stimulation, amplitude of unloaded cell shortening increased with increasing frequency of stimulation (0.04 ± 0.01 $\Delta L/L_0$ at 1 Hz to 0.07 ± 0.01 $\Delta L/L_0$ at 4 Hz, $n = 12$, $P < 0.05$). During whole cell voltage clamp with 50 μM $[K5\text{-fluo-3}]_{pip}$, both peak and baseline $[Ca^{2+}]_i$ increased at higher stimulation frequencies, but the net $\Delta[Ca^{2+}]_i$ increased only modestly from 1.59 ± 0.08 $\Delta F/F_0$ at 1 Hz, to 1.71 ± 0.11 $\Delta F/F_0$ at 4 Hz ($n = 17$, $P < 0.05$). When a 1 s pause was interposed during stimulation at 2 and 4 Hz, $[Ca^{2+}]_i$ transients were significantly larger (at 4 Hz, peak F/F_0 increased by 78 ± 2 %, $n = 5$). SR Ca^{2+} content assessed during caffeine application, significantly increased from 91 ± 24 μmol l^{-1} at 1 Hz to 173 ± 20 μmol l^{-1} at 4 Hz ($n = 5$, $P < 0.05$). Peak $I_{Ca,L}$ decreased at higher frequencies (by 28 ± 6 % at 2 Hz, and 45 ± 8 % at 4 Hz), due to slow recovery from inactivation. This loss of $I_{Ca,L}$ resulted in reduced fractional release. Thus, in mouse ventricular myocytes the $[Ca^{2+}]_i$–frequency response depends on a balance between the increase in SR content and the loss of trigger $I_{Ca,L}$. Small changes in this balance may contribute to variability in frequency-dependent behaviour. In addition, there may be a regulation of the contractile response downstream of $[Ca^{2+}]_i$.

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The change in contractile force in response to a change in frequency of stimulation is a general property of cardiac muscle. For most species, including healthy humans, the relation is a positive one, i.e. force of contraction increases with increasing frequency of stimulation (e.g. Gwathmey *et al.* 1990 for human). This positive staircase results from larger $[Ca^{2+}]_i$ transients at higher frequencies, due to an increase in sarcoplasmic reticulum (SR) content with increasing stimulation frequency (reviewed in Bers, 2001). Frequency-dependent facilitation of $I_{Ca,L}$ may also contribute to the positive staircase (Zygmunt & Maylie, 1990). For the rat the relation is negative in the lower range of frequencies (below 2 Hz, e.g. Henderson *et al.* 1974; but see also Layland & Kentish, 1999). It is thought that resting SR content in the rat is high and cannot be increased in this range of frequencies; incomplete recovery of the release channel during stimulation at higher frequencies would then result in a negative staircase (reviewed in Bers, 2001). The force–frequency relation is often used to describe the contractile state and can be altered by inotropic interventions, e.g. by drugs that increase $[Na^+]_i$ (Koch-Weser & Blinks, 1963; Mubagwa *et al.* 1997) or that affect SR Ca^{2+} uptake and release (Baudet *et al.* 1996; Bers & Christensen, 1990), and by disease states such as heart failure (Mulieri *et al.* 1992). Changes in the relation usually reflect alterations in one of the underlying mechanisms, e.g. the diminished capacity of the SR to increase its Ca^{2+} content, which has been implicated in the negative force–frequency of the failing human heart (Pieske *et al.* 1999).

Because of transgenic technology, the mouse has become a much-studied species for cardiovascular pathophysiology. The basic, normal physiology, may however be quite particular in this small animal with high *in vivo* heart rates. For the frequency response, experiments on trabeculae have shown a positive relation with an increase in force for increase in stimulation frequency from 1 to 4 Hz (Gao *et al.* 1998). The response in the amplitude of the Ca^{2+} transient was also positive, though less pronounced than for contraction. *In vivo*, the frequency response appears to be rather flat in the range from 500 to 850 beats min^{-1} (Georgakopoulos & Kass, 2001). In some of the recent transgenic mouse models the frequency response was found to be altered, e.g. by changes in phospholamban, PLB, expression (Kadambi *et al.* 1999), or by knockout of Annexin VII (Herr *et al.* 2001). Yet the mechanisms

determining the frequency response in the mouse, which may be particular to this species, have not been studied. We therefore examined the characteristics of the frequency response of contraction and of Ca^{2+} fluxes in single mouse ventricular myocytes. We found a prominent role for frequency-dependent reductions in $I_{Ca,L}$ which offset the effect of an increase in SR Ca^{2+} content at higher frequencies. This interplay between factors promoting a positive frequency response and factors promoting a negative frequency response results in a rather flat $[Ca^{2+}]_i$–frequency relation.

METHODS

Cell isolation

Single ventricular myocytes were enzymatically dissociated from 3- to 4-month-old mice (strain 129/SV). Mice were killed by an overdose of pentobarbital (100 mg kg^{-1} I.P.), and the heart quickly excised. After cannulation of the aorta, hearts were mounted to a Langendorff perfusion set. The heart was briefly rinsed with normal Tyrode solution, containing (mM): 137 NaCl, 5.4 KCl, 0.5 $MgCl_2$, 1 $CaCl_2$, 11.8 Hepes and 10 glucose, pH adjusted to 7.4 with NaOH. Subsequently it was perfused with a Ca^{2+}-free solution for 8 min. The Ca^{2+}-free Tyrode solution contained (mM): 130 NaCl, 5.4 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 6 Hepes, 20 glucose, pH adjusted to 7.2 with NaOH. Collagenase A (0.6 mg ml^{-1}, Roche Diagnostics, GmbH, Mannheim, Germany) and protease (type XIV, 0.1 mg ml^{-1}, Sigma, St Louis, MO, USA), added to the Ca^{2+}-free solution, were recirculated for 10 min. The enzymes were washed out with low Ca^{2+} solution, i.e. the Ca^{2+}-free solution to which 0.18 mM $CaCl_2$ was added. The heart was then removed, the ventricles were cut into small pieces and further dissociated into single cells by gentle shaking. Cells were stored in low Ca^{2+} solution at room temperature and used within 8 h after isolation. All experimental procedures were approved by the Ethics Committee on Animal Use of the University of Leuven.

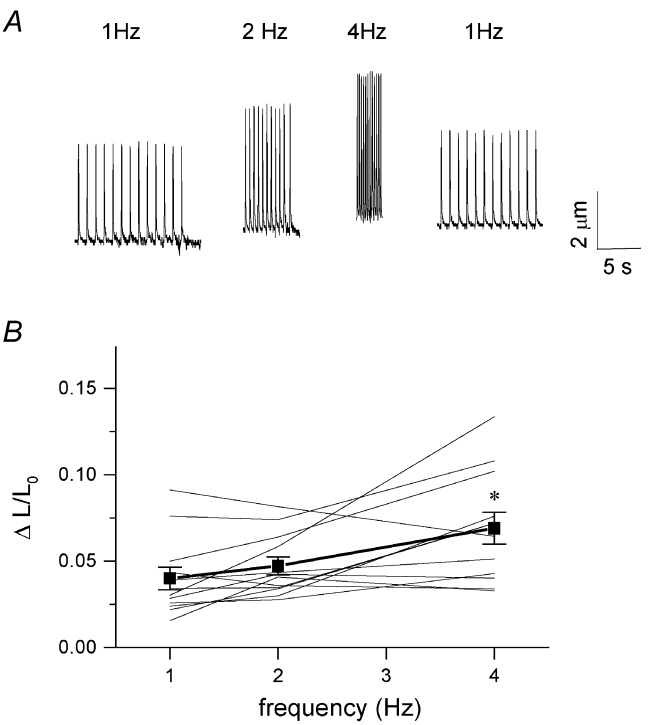

Figure 1. Frequency dependence of cell shortening in mouse myocytes

A, example of cell shortening measurements in an unloaded cell, externally stimulated at 1, 2 and 4 Hz, showing a positive frequency response. *B*, frequency response of amplitude of cell shortening (ΔL), normalized to resting cell length (L_0) of individual cells, represented by thin lines, one cell showed a negative response. Average data show an increase in amplitude with increasing stimulation frequency (mean ± S.E.M., $n = 12$, * $P < 0.05$ *vs.* 1 Hz).

Measurements of contraction, $[Ca^{2+}]_i$ and membrane currents

The setup for combined $[Ca^{2+}]_i$ and membrane current recording was as described before (Sipido *et al.* 1997).

Cell shortening was measured with a video-edge detector (Crescent, Salt Lake City, UT, USA) at 240 Hz frame rate. Field stimulation was done with 5 ms square pulses of constant voltage, at 20 % above threshold. The absolute cell shortening is expressed as the fractional shortening, i.e. normalized to resting cell length, $\Delta L/L_0$.

$[Ca^{2+}]_i$ was measured with fluo-3, and is reported as the fluorescence normalized to baseline values, after background subtraction, F/F_0. For higher frequencies of stimulation the baseline fluorescence increased, and values were normalized to the value at rest, before the onset of stimulation, or 10 s after stopping stimulation. In a subset of voltage clamped cells we calibrated fluo-3 signals after obtaining the F_{max}, as described by Trafford *et al.* (1999). With this method we established that $[Ca^{2+}]_i$ of resting myocytes is 108 ± 10 nM ($n = 8$). To calibrate signals of field-stimulated cells, loaded with fluo-3-AM, we used the approach of Cheng *et al.* (1993) assuming a K_d of 800 nM and a resting $[Ca^{2+}]_i$ of 108 nM.

Membrane currents were sampled at 4 kHz and filtered at 1 kHz. For the global analysis of frequency dependence of Ca^{2+} release, we used a K^+-based pipette solution (mM): 120 potassium aspartate, 20 KCl, 10 Hepes, 5 MgATP, 10 NaCl, 0.05 K5-fluo-3, pH adjusted to 7.2 using KOH, and Tyrode solution with 1 mM $CaCl_2$ as the external solution. For measurements of L-type Ca^{2+} current, we blocked K^+ currents and Cl^- concentrations were set to have a reversal potential near 0 mV. For these experiments the pipette solution contained (mM): 130 CsCl, 10 Hepes, 0.5 $MgCl_2$, 4 MgATP (pH 7.2 with CsOH), and the external solution contained (mM): 130 NaCl, 10 CsCl, 11.8 Hepes, 0.5 $MgCl_2$, 1 $CaCl_2$, 10 glucose, with pH 7.4 with NaOH.

To determine SR Ca^{2+} content, we perfused the cell with 10 mM caffeine for 8 s to empty the stores and extrude Ca^{2+} via Na^+–Ca^{2+} exchange. The Na^+–Ca^{2+} exchange current was integrated and from the resulting charge we recalculated the amount of Ca^{2+} released from the SR (Varro *et al.* 1993). We applied a correction for Ca^{2+} removal by other pathways of 19 %. This value is based on measurements of the rate of decline of $[Ca^{2+}]_i$ in the presence of caffeine and with Na^+–Ca^{2+} exchange blocked with $NiCl_2$ (Varro *et al.* 1993). The SR Ca^{2+} content is expressed as $[Ca^{2+}]$ per accessible cell volume, assuming a surface/volume (*S/V*) ratio of 7.47 pF pl^{-1}, and an accessible fraction of 0.65. The *S/V* is based on measurements of cell capacity and estimates of cell volume during confocal imaging as described by Satoh *et al.* (1996). The values are close to those reported for rat ventricular myocytes, a species which like the mouse has an extensive T-tubular network, and are consistent with morphology data from the literature (reviewed in Bers, 2001).

All experiments were done at 30 °C. Different frequencies were tested in random order to minimize time-dependent changes. For statistical analysis we used ANOVA for repeated measurements, with Bonferroni or Tukey *post hoc* testing; values of $P < 0.05$ were considered significant. Values are reported as mean ± S.E.M.

RESULTS

Frequency dependence of contraction of unloaded myocytes

Figure 1 shows in the top panel original traces of cell shortening in a myocyte stimulated at 1, 2 and 4 Hz. The amplitude of shortening increased with increasing frequency, but the cell did not relax completely at 2 and 4 Hz. The lower panel summarizes the data for 12 cells. Only one cell has a frank negative response. A few cells have a very pronounced positive response. On average there is a significant increase in the amplitude of shortening at higher stimulation frequencies: $\Delta L/L_0$ increases from 0.040 ± 0.007 at 1 Hz, to 0.047 ± 0.005 at 2 Hz, and to 0.069 ± 0.009 at 4 Hz ($P < 0.05$).

Frequency dependence of $[Ca^{2+}]_i$ transients

We further studied the underlying mechanisms of the frequency response in whole cell voltage clamp mode. The mouse action potential is very brief and in current clamp mode we found that at 25 ms the membrane had repolarized to values below −50 mV ($n = 8$, data not shown). In the voltage clamp mode we thus used 25 ms depolarizing steps from −70 to +20 mV. Figure 2*A* shows in the top panel a typical example of $[Ca^{2+}]_i$ transients during this protocol. The peak $[Ca^{2+}]_i$ increases with frequency, and so does the baseline $[Ca^{2+}]_i$. The data for all cells is summarized in Fig. 2*B*. Baseline $[Ca^{2+}]_i$, expressed as F/F_0, increases from 1.03 ± 0.01 at 1 Hz to 1.25 ± 0.06 at 2 Hz, and to 1.60 ± 0.10 at 4 Hz; peak $[Ca^{2+}]_i$ increases from 2.58 ± 0.08 at 1 Hz to 2.94 ± 0.11 at 2 Hz, and to 3.31 ± 0.08 at 4 Hz ($n = 17$, $P < 0.05$). In panel *C*, the average data for the amplitude of the $[Ca^{2+}]_i$ transients, i.e. peak − baseline, is shown. On average there is a small increase in the amplitude: $\Delta F/F_0$ increases from 1.56 ± 0.08 at 1 Hz to 1.70 ± 0.11 at 2 Hz and 1.71 ± 0.11

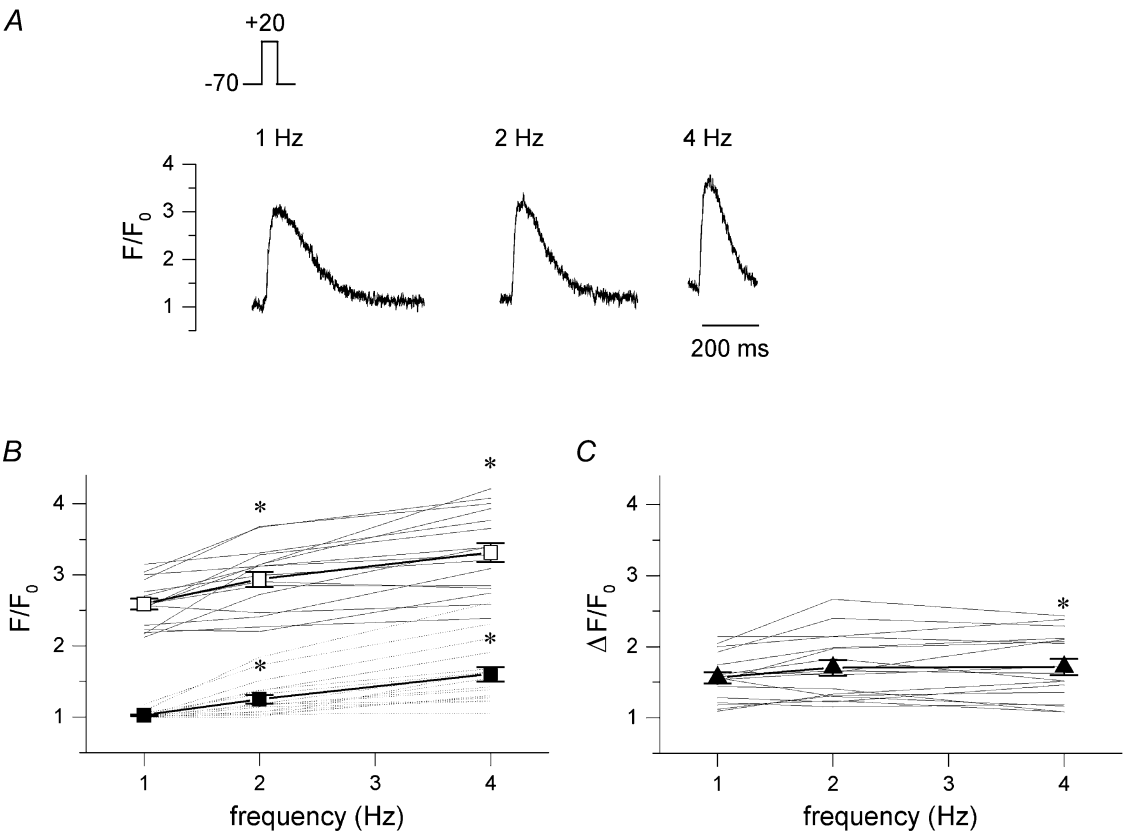

Figure 2. Frequency dependence of $[Ca^{2+}]_i$ transients

A, original current traces, *I*, and $[Ca^{2+}]_i$ transients, F/F_0, recorded in a voltage clamped cell during steady-state stimulation with 25 ms depolarizing steps from −70 to +20 mV at the indicated frequencies. *B*, individual data for frequency dependence of $[Ca^{2+}]_i$ transients, F/F_0, showing peak values (continuous lines) and baseline values (dashed lines) of 17 cells. Pooled data show an increase in peak $[Ca^{2+}]_i$ (□) and baseline $[Ca^{2+}]_i$ (■) at higher stimulation frequencies (mean ± S.E.M., * $P < 0.05$ *vs.* 1 Hz). *C*, amplitude of the $[Ca^{2+}]_i$ transients, $\Delta F/F_0$, with increasing stimulation rate (mean ± S.E.M., * $P < 0.05$ *vs.* 1 Hz).

at 4 Hz ($n = 17$, $P < 0.05$ for 4 Hz only). This change in amplitude is modest, suggesting there is little increase in SR Ca^{2+} release.

We checked that this was not due to the rather short pulses. In three cells, we compared the frequency response with 25 ms pulses to the response with 100 ms pulses. The absolute $[Ca^{2+}]_i$ values were larger for the 100 ms pulses, consistent with higher SR content for longer pulse duration (Terracciano *et al.* 1997), but the relative change with frequency was the same (data not shown).

The lack of significant increase in Ca^{2+} release at higher frequencies could be the result of a lack of increase in SR Ca^{2+} content, and/or of a decrease in fractional release due to a decrease in (trigger) Ca^{2+} current and/or opening of the Ca^{2+} release channel, the ryanodine receptor, RyR. In the first case, introducing a longer pause during the stimulation will not increase SR Ca^{2+} release, but in the second case a pause will increase SR Ca^{2+} release, as it may relieve inactivation of $I_{Ca,L}$ and/or RyR. We thus tested these hypotheses in the experiment illustrated in Fig. 3*A*. Following a train of pulses at 2 and 4 Hz, we introduced a 1 s pause before the next depolarizing test step. The $[Ca^{2+}]_i$ transients with this test step, *t*, were compared to the last $[Ca^{2+}]_i$ transient of the conditioning step, *c*. In the example it can be seen that the $[Ca^{2+}]_i$ transients for *t* are significantly larger than for *c* at 2 Hz and at 4 Hz, suggesting there is a decrease in $I_{Ca,L}$ and/or ryanodine receptor availability. Average data are shown in Fig. 3*B*, and the differences between test and conditioning pulses are significant (# $P < 0.05$, $n = 5$). In this same experiment we also compared the test steps at 2 and 4 Hz with the steady-state at 1 Hz. This analysis shows that for *t* there is a large increase with frequency (* $P < 0.05$), suggesting that there is an increase in SR Ca^{2+} content during the conditioning pulses.

SR Ca^{2+} content at different frequencies of stimulation

The SR content was measured by emptying the SR with a fast application of 10 mM caffeine following conditioning pulses to the different frequencies. A typical example is shown in Fig. 4*A*, with $[Ca^{2+}]_i$ transients shown as F/F_0 and the membrane current shown as the running integral. Average values are given in panels *B* and *C*. Peak of caffeine-induced transients increases with increasing frequency of stimulation (peak F/F_0 from 4.86 ± 0.34 at 1 Hz, to 5.86 ± 0.30 at 2 Hz and to 6.75 ± 0.40 at 4 Hz

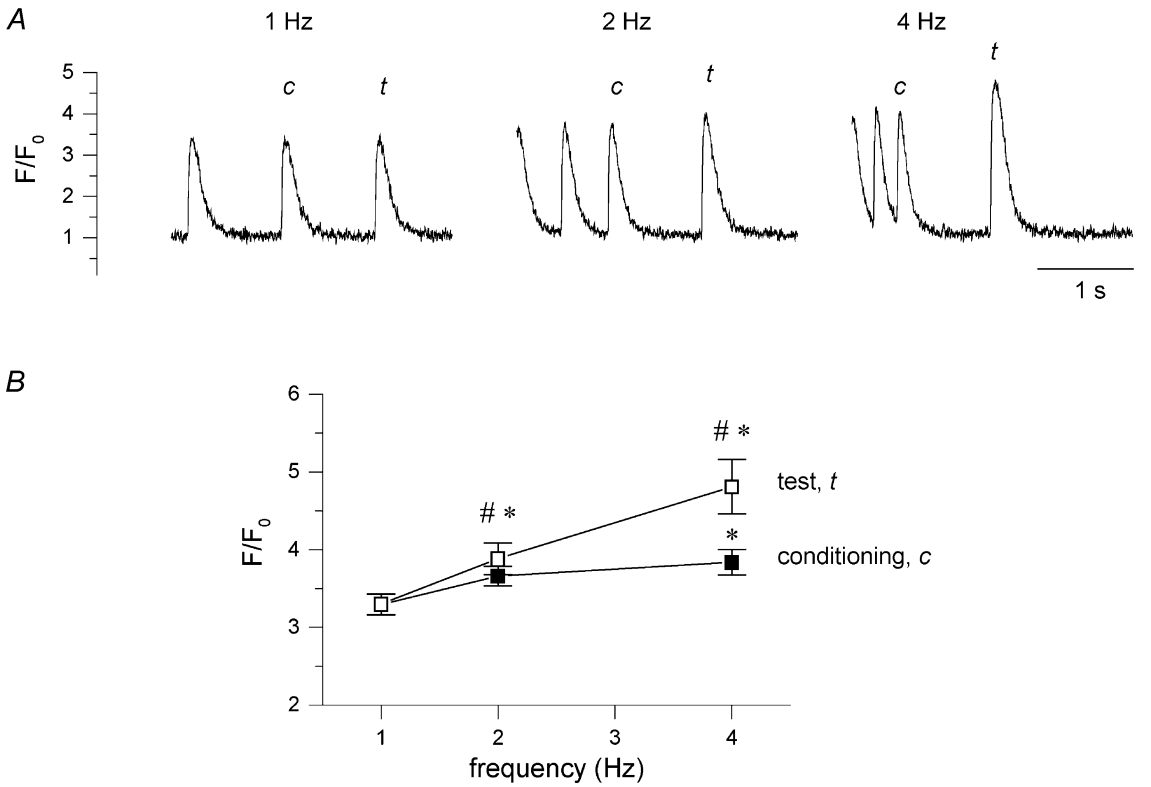

Figure 3. $[Ca^{2+}]_i$ transients following 1 s pause after different stimulation frequencies

A, typical example of $[Ca^{2+}]_i$ transients, F/F_0, elicited by a 25 ms depolarizing pulse from −70 to +20 mV following 1 s interval (test pulse, *t*) after stimulation with 10 conditioning pulses (conditioning, *c*) at different frequencies of 1, 2 and 4 Hz. *B*, pooled data of peak $[Ca^{2+}]_i$, F/F_0, of the test pulse (□) and of the last conditioning pulse (■) as a function of increasing stimulation frequency (# $P < 0.05$ for *t vs. c*, * $P < 0.05$ for 2 and 4 Hz *vs.* 1 Hz, mean ± S.E.M., $n = 5$).

($n = 5$, $P < 0.05$). SR Ca^{2+} content, calculated from $I_{Na,Ca}$ as described in Methods, increases from 91 ± 24 μM at 1 Hz to 132 ± 17 at 2 Hz and 173 ± 20 μM at 4 Hz ($n = 5$, $P < 0.05$).

In Fig. 5*A*, we compared the amplitude of the stimulation-induced $[Ca^{2+}]_i$ transient to the amplitude of the caffeine-induced $[Ca^{2+}]_i$ transient, as a rough approximation of fractional release during stimulation at 1, 2 and 4 Hz. For this we divided the amplitude of the last $[Ca^{2+}]_i$ transient of the conditioning train, $\Delta F/F_0$ of *c*, by the amplitude of the caffeine transient, $\Delta F/F_0$ of caffeine, evoked at the end of the train. This value decreases from 49 ± 5 % at 1 Hz, to 41 ± 4 % at 2 Hz and to 31 ± 5 % at 4 Hz ($n = 5$, $P < 0.05$). In Fig. 5*B*, we have plotted the ratio of the amplitude of the last $[Ca^{2+}]_i$ transient of the conditioning train, $\Delta F/F_0$ of *c*, over the amplitude of the test pulse transient, $\Delta F/F_0$ of *t* (data from Fig. 3). This ratio decreases with frequency, in a manner very similar to the ratio with the caffeine-induced $[Ca^{2+}]_i$ transient. These data are consistent with the hypothesis that there is a decrease in the trigger for SR Ca^{2+} release, or in the availability of the RyR.

Changes in $I_{Ca,L}$ with increasing frequency of stimulation

To measure $I_{Ca,L}$ we used other solutions than in the previous experiments to exclude contamination by K^+ and Na^+ currents (see Methods). The test pulse was set to 0, instead of to +20 mV, to prevent a potential contribution of Cl^- currents. Figure 6*A* shows the time course of a typical experiment, recording the amplitude of $I_{Ca,L}$ during different frequencies. During each series of pulses there is decrease in peak current, which is largest for the highest frequencies. The current recovers during rest periods in between trains of stimulation at the various frequencies, excluding that this results from rundown of channels. In Fig. 6*B*, the average loss of peak current for the three frequencies tested is shown. $I_{Ca,L}$ is reduced to 88 ± 3 % at 1 Hz, 72 ± 6 % at 2 Hz and 54 ± 8 % at 4 Hz ($n = 6$, $P < 0.05$). These data suggest that there is inactivation and incomplete recovery of $I_{Ca,L}$ at the higher frequencies.

This was tested more directly during an inactivation-recovery protocol as illustrated in Fig. 6*C*. At the longest interval of 500 ms, recovery is nearly, but not fully

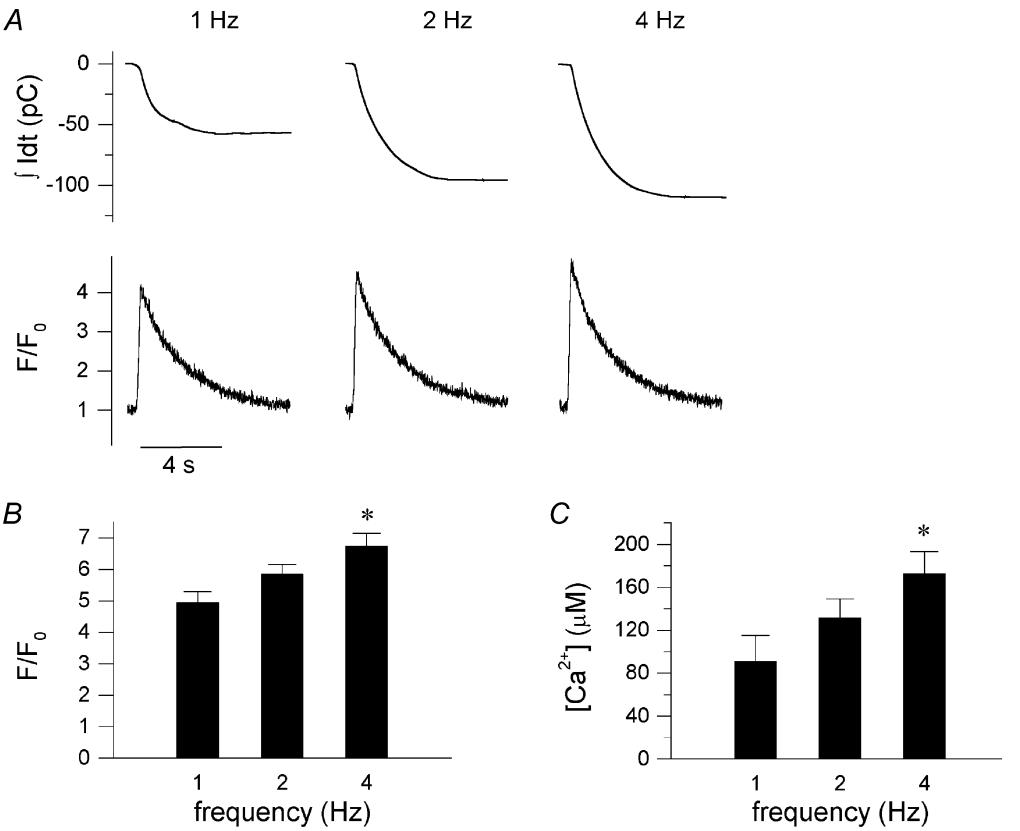

Figure 4. Frequency dependence of SR Ca^{2+} content

A, typical example of integrated Na^+–Ca^{2+} exchange current, shown as the running integral, and $[Ca^{2+}]_i$ transients, F/F_0, induced by 8 s application of 10 mM caffeine following a 1 s pause after stimulation with 10 conditioning pulses at the indicated frequency (1, 2 and 4 Hz). *B*, peak of caffeine-induced transients, F/F_0, with increasing frequency of stimulation (mean ± S.E.M., $n = 5$, $P < 0.05$). *C*, SR Ca^{2+} content, calculated from integrating $I_{Na,Ca}$, and normalized to accessible cell volume, as described in the Methods section (mean ± S.E.M., $n = 5$, $P < 0.05$).

complete. On average $I_{Ca,L}$ recovery was 84 ± 3 % at 250 ms and 95 ± 3 % at 500 ms (n = 7). In the example shown, recovery of the $[Ca^{2+}]_i$ transient exceeds the time for recovery of the current, suggesting that release itself may recover more slowly. On average, the recovery of the amplitude of the $[Ca^{2+}]_i$ transient, $\Delta F/F_0$, was 38 ± 6 % at 250 ms and 83 ± 6 % at 500 ms.

We also calculated the impact of the reduction of $I_{Ca,L}$ at higher frequencies on the total Ca^{2+} influx. Using the steady-state data from experiments as shown in Fig. 6, we calculated that the Ca^{2+} influx via $I_{Ca,L}$ was 3.34 ± 0.65 μm l^{-1} s^{-1} at 1 Hz, 5.40 ± 1.09 μm l^{-1} s^{-1} at 2 Hz and 8.06 ± 1.90 μm l^{-1} s^{-1} at 4 Hz (n = 6, normalized to accessible cell volume).

Frequency dependence of decline of $[Ca^{2+}]_i$

To study the changes in relaxation at different frequencies, we measured the half-time of decay of the $[Ca^{2+}]_i$ transient at steady-state stimulation. Because the relaxation is incomplete at the higher frequencies, we measured half-time of decay for the last pulse of a train at each frequency. At 1 Hz the half-time is 126 ± 18 ms, at 2 Hz it is 113 ± 13 ms, and at 4 Hz, 105 ± 9 ms. These data indicate that Ca^{2+} removal is indeed faster at the higher frequencies.

Relation between the frequency dependence of Ca^{2+} release and of contraction

Comparing the frequency dependence of contractions measured in field-stimulated myocytes (Fig. 1), with the frequency dependence of $[Ca^{2+}]_i$ transients measured in voltage clamped cells (Fig. 2), it seems that the contraction–frequency relation is more steep than the $[Ca^{2+}]_i$–frequency relation. Since these were independent measurements with possible confounding factors, we loaded cells with the ester form of fluo-3 and recorded simultaneously $[Ca^{2+}]_i$ transients and cells shortening in field-stimulated cells. In these cells, as in Fig. 1*B*, positive, flat and negative relations were observed. In Fig. 7 we have pooled the data of eight cells with a positive contraction–frequency response, together with the $[Ca^{2+}]_i$–frequency relation. These data support the impression from the previous independent measurements, namely that the increase in contraction amplitude is more pronounced than the increase in the $[Ca^{2+}]_i$ transient amplitude. Compared to 1 Hz, shortening amplitude, $\Delta L/L_0$, increases approximately 1.2- and 1.8-fold at 2 and 4 Hz respectively, whereas $\Delta[Ca^{2+}]_i$ increases 1.1- and 1.4-fold.

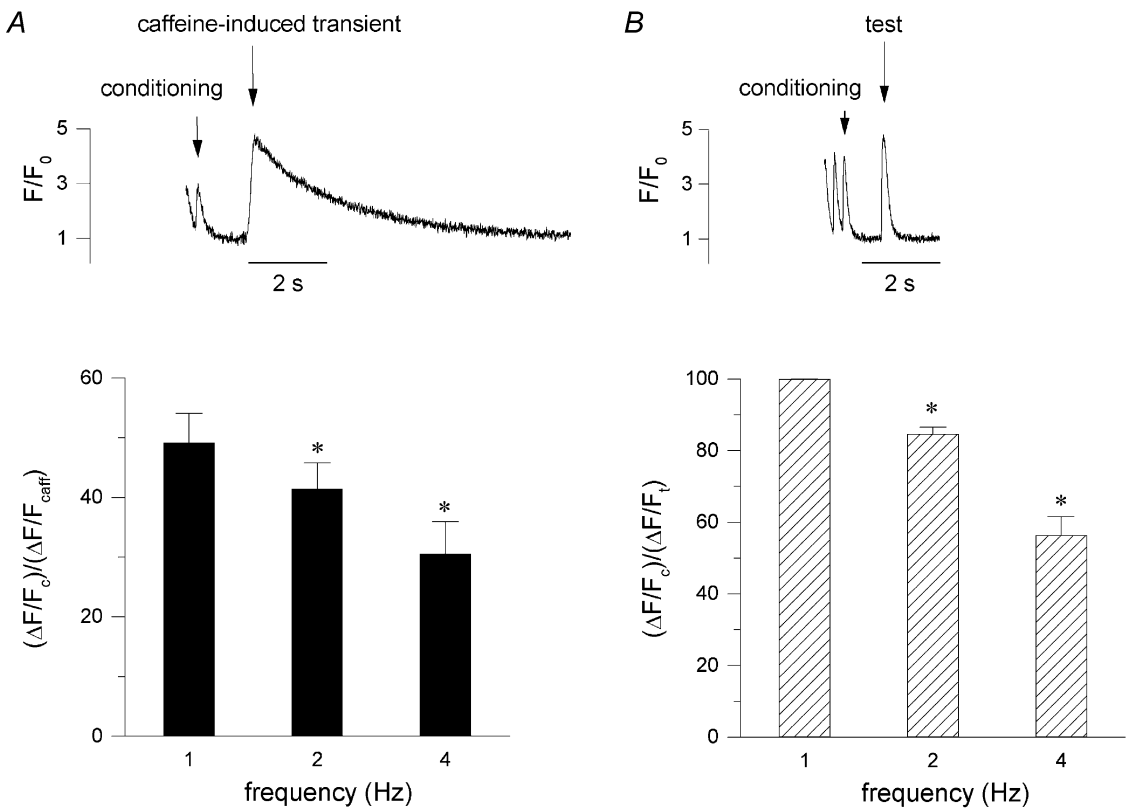

Figure 5. Reduced 'fractional' release at higher frequencies

A, the ratio of the amplitude of the $[Ca^{2+}]_i$ transients of the conditioning pulses, $\Delta F/F_c$, and the $[Ca^{2+}]_i$ transient of caffeine-induced transient $\Delta F/F_{caff}$, from experiments as shown in the inset, as an estimate of fractional release (mean ± S.E.M., n = 5, $P < 0.05$). *B*, for comparison we also calculated the ratio between the amplitude of the $[Ca^{2+}]_i$ transients of the conditioning pulses, $\Delta F/F_c$, relative to the $[Ca^{2+}]_i$ transient of the test pulse after a 1 s pause, $\Delta F/F_t$, at different frequencies (mean ± S.E.M., n = 5, $P < 0.05$). Data from the same experiments as shown in Fig. 3.

DISCUSSION

In the mouse, the increase in $[Ca^{2+}]_i$ transient amplitude with increasing frequencies of stimulation is modest. An unexpected and novel finding was that the frequency-dependent increase in SR content is actually very steep. At the higher frequencies of stimulation a loss of trigger for release offsets this increase in SR Ca^{2+} content, resulting in the rather shallow $[Ca^{2+}]_i$–frequency response. This loss of trigger with increasing frequency has not been reported before. We also observed that the increase in contraction amplitude is disproportionately larger than the increase in the $[Ca^{2+}]_i$ transient, suggesting that there may be a regulation downstream of $[Ca^{2+}]_i$.

Increase in SR content with increasing frequency of stimulation

The increase in SR content is consistent with reports in other animal species, including healthy humans. The underlying mechanism must be Ca^{2+} influx exceeding the Ca^{2+} efflux. We calculated that net influx via $I_{Ca,L}$ is indeed larger at the higher frequencies, despite the reduction in peak current. A reduction of Ca^{2+} efflux via the Na^+–Ca^{2+} exchanger is due to a shorter time spent at diastolic membrane potentials, and to the increase in $[Na^+]_i$ at higher frequencies (e.g. Cohen *et al.* 1982; Frampton *et al.* 1991; Harrison & Boyett, 1995), which would shift the reversal potential of the exchanger to more negative values. On the other hand, the increase in diastolic $[Ca^{2+}]_i$ would counteract this shift. In preliminary experiments on mouse myocytes we observed an increase in cytosolic $[Na^+]$ in mouse myocytes, but the increase was rather slow compared to the increase in contraction (F. Moccia & K. Mubagwa, unpublished observations). Subsarcolemmal gradients may be more important for shifts in reversal potential of the Na^+–Ca^{2+} exchange, as suggested by recent studies (Su *et al.* 2001).

Another potential mechanism for the increase in SR content is increased sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA) activity due to Ca^{2+}/calmodulin kinase phosphorylation of PLB (Bassani *et al.* 1995; Hagemann *et al.* 2000) or through other pathways (Li *et al.* 1998). Our

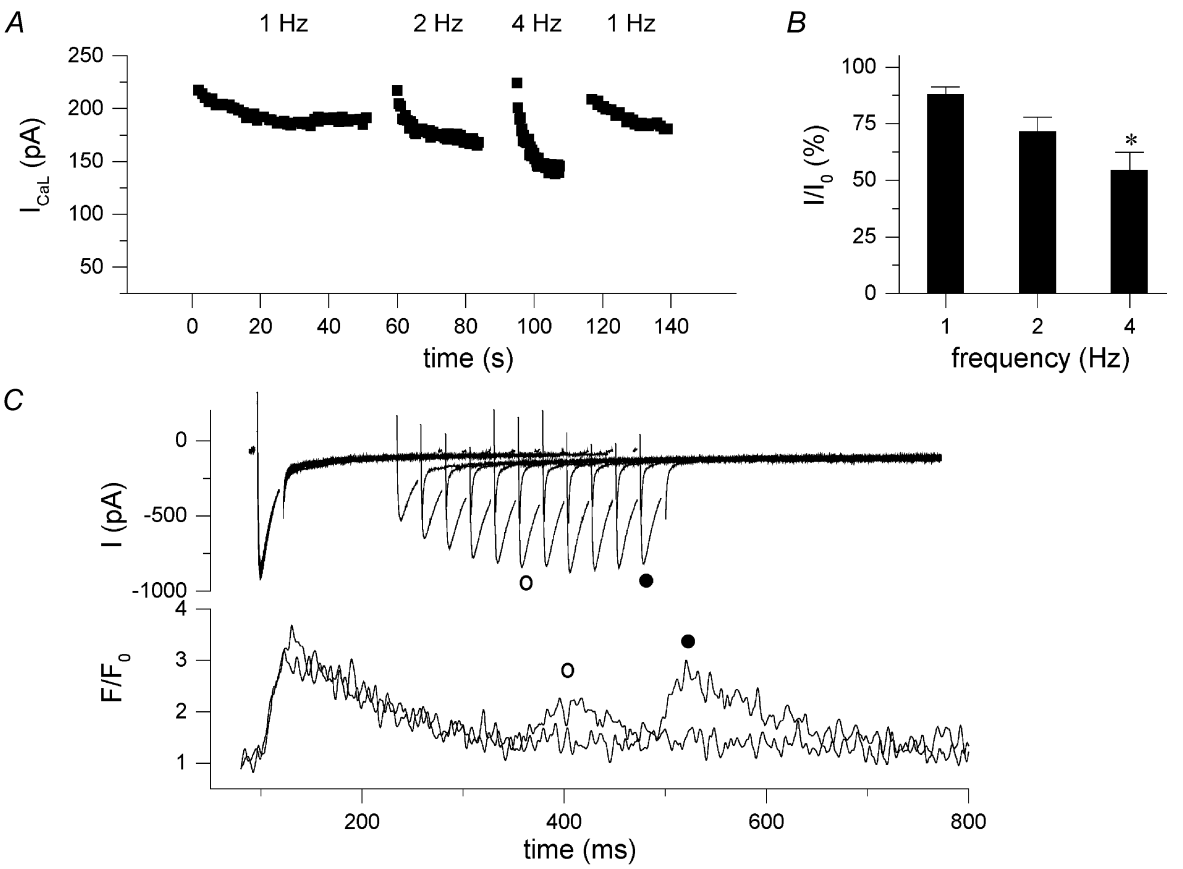

Figure 6. Frequency dependence of L-type Ca^{2+} current

A, time course of a typical experiment measuring $I_{Ca,L}$ during stimulation at 1, 2 and 4 Hz with a 10 s interval between the different frequencies, showing absolute peak values of $I_{Ca,L}$ at each frequency. $I_{Ca,L}$ was measured with 25 ms depolarizing steps from −70 to 0 mV in K^+-free internal and external solutions. *B*, peak of steady-state current, expressed as percentage of I_0 (mean ± S.E.M., $n = 6$). *C*, recovery from inactivation of $I_{Ca,L}$; a 25 ms depolarizing step from −70 to +20 mV was followed by a test step after a 125 ms interval, and this interval was increased by 25 ms steps, interpulse duration was 10 s.

results are also consistent with a faster SR uptake at higher frequencies.

Lastly, the decrease in $I_{Ca,L}$ at higher frequency and the decrease in fractional release will reduce the Ca^{2+} efflux via the exchanger and reduce Ca^{2+}-dependent inactivation of $I_{Ca,L}$, thus increasing SR content (Trafford *et al.* 2001). This and the above mentioned mechanisms are likely to act in concert to increase SR content.

Loss of $I_{Ca,L}$ at higher frequencies

In the mouse, the loss of Ca^{2+} current with stimulation at 2 and 4 Hz is apparently related to slow recovery from inactivation. In our experiments $[Ca^{2+}]_i$ was not buffered and Ca^{2+}-dependent mechanisms are likely to be of major importance, as probably also *in vivo*. The decay of $[Ca^{2+}]_i$ is not complete at 250 ms, as can be seen in Fig. 6, and at steady-state 4 Hz stimulation, diastolic $[Ca^{2+}]_i$ is clearly elevated. The time course of beat-to-beat decrease in $I_{Ca,L}$ does however not occur in parallel with a beat-to-beat increase in diastolic $[Ca^{2+}]_i$. The increase in diastolic $[Ca^{2+}]_i$ indeed occurs rather abruptly with the first short stimulation interval (data not shown). $[Ca^{2+}]_i$ remains then elevated at the same level, whereas the Ca^{2+} current continues to decline. One explanation is the accumulation of inactivation, as recovery is incomplete. Another factor may be that the subsarcolemmal $[Ca^{2+}]$ is more elevated.

The pronounced decrease of $I_{Ca,L}$ with frequency in normal mouse myocytes has not yet been described. We have however previously observed a loss of $I_{Ca,L}$ with frequent stimulation in myocytes from failing human hearts, where it will contribute to the negative staircase typical of heart failure (Sipido *et al.* 1998). The loss of $I_{Ca,L}$ in the human failing heart occurs at lower frequencies of stimulation (0.5, 1 and 2 Hz), but is also due to slow recovery from inactivation. In the case of the failing heart, the slow removal of Ca^{2+} by the SR has a role in the current inactivation. This loss of $I_{Ca,L}$ leads to a decrease in the plateau values of the action potential. Li *et al.* (1999) have also observed a loss of $I_{Ca,L}$ at higher frequencies of stimulation in human myocytes from the right ventricle (RV) of failing hearts. These authors could show that shortening of the action potential at these higher frequencies is the result of a decrease in $I_{Ca,L}$. The decrease in $I_{Ca,L}$ at higher frequencies in patients is an important mechanism in the negative staircase, and helps to explain the beneficial effects of slowing the heart rate. Our observations in the mouse indicate that this phenomenon may more common than we thought, and may be present in healthy cardiac muscle.

The combination of a reduced $I_{Ca,L}$ and increasing SR Ca^{2+} content at higher frequencies predicts that the effect of pause as e.g. after an ectopic beat, may have major consequences for both the action potential time course and the amplitude of contraction, because of enhanced recovery of $I_{Ca,L}$ and increased Ca^{2+} release. This mechanism could be important for arrhythmogenesis.

Our findings of a pronounced loss of $I_{Ca,L}$ contrasts with several reports on frequency-dependent potentiation of $I_{Ca,L}$, which also appears to be Ca^{2+}-dependent (see e.g. Zygmunt & Maylie, 1990; Bates & Gurney, 1993; and review in Anderson, 2001). Probably both potentiation and inhibition can occur, and a potential explanation is that both phenomena have a different sensitivity to Ca^{2+}. Potentiation is usually observed with small increases in $[Ca^{2+}]_i$ when there is moderate $[Ca^{2+}]_i$ buffering, e.g. with EGTA (Fedida *et al.* 1988*a*; Hryshko & Bers, 1990;

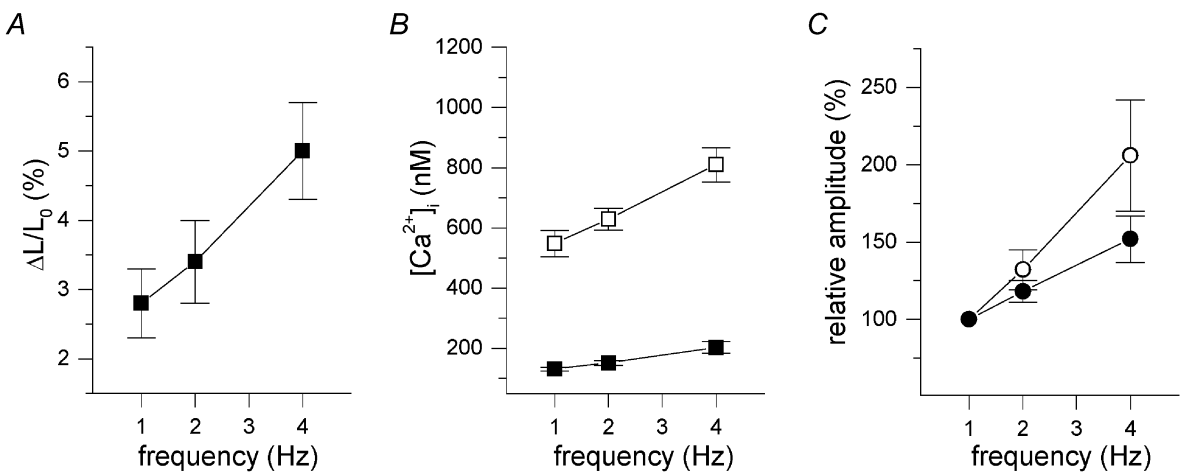

Figure 7. Simultaneous measurement of cell shortening and $[Ca^{2+}]_i$ transients during field stimulation

Pooled data of eight cells loaded with fluo-3AM, and calibrated $[Ca^{2+}]_i$ signals. *A*, amplitude of fractional cell shortening. *B*, peak (□) and basal (■) $[Ca^{2+}]_i$ of transients. *C*, relative amplitude of cell shortening (○), calculated as percentage of the amplitude at 1 Hz, compared to the relative increase in the amplitude of the $[Ca^{2+}]_i$ transients (●).

Zygmunt & Maylie, 1990). Without $[Ca^{2+}]_i$ buffering and with larger increases in $[Ca^{2+}]_i$ inhibition occurs (Fedida *et al.* 1988*b*). The mechanisms of such a double response remain however elusive. Recent mutational analysis of the $\alpha 1$ subunit identified Ca^{2+}/calmodulin binding sites, but the same sites were involved in facilitation as well as inactivation (Zuhlke *et al.* 2000).

Our data cannot exclude that slow recovery from inactivation of the RyR (reviewed in Bers, 2001) also contributes to the reduced fractional release at higher frequencies. The data of Fig. 6 could be interpreted this way, as Ca^{2+} release recovers more slowly than $I_{Ca,L}$. However, as we compare the recovery to a first beat after a 10 s pause, it is possible that the SR Ca^{2+} content is not the same. We therefore cannot establish with certainty the role of RyR recovery.

Relation between the $[Ca^{2+}]_i$ and contraction response to frequency

An additional finding was that contraction increased more steeply with frequency, than the amplitude of the $[Ca^{2+}]_i$ transient. This is consistent with the previous report by Gao *et al.* (1998) who measured simultaneously $[Ca^{2+}]_i$ and isometric contractions in trabeculae of mouse hearts at room temperature. A phase-plane analysis of force *vs.* $[Ca^{2+}]_i$ showed that the relation between $[Ca^{2+}]_i$ and force was dependent on the stimulation frequency, and steeper at higher frequencies. This is an unusual finding not reported in other animal species. The 'sensitization' of myofilaments at higher frequencies could be related to the increase in diastolic $[Ca^{2+}]_i$ which may affect phosphorylation of myosin light chains (Morano *et al.* 1990). The lack of full relaxation of the cell at higher frequencies may be related to such a sensitization phenomenon. Another explanation is that the shift in diastolic $[Ca^{2+}]_i$ moves the starting point of the contraction more towards the steep part of the tension–pCa relation. Indeed, the tension–pCa reported by Gao *et al.* (1998) has a shallow foot, but is then very steep for $[Ca^{2+}]_i$ above 500 nM. Although calibration of fluo-3 is not as reliable as for fura-2, our estimate is that basal $[Ca^{2+}]_i$ increases from 108 ± 10 nM to 208 ± 30 nM ($n = 10$) at 4 Hz. This is still below the activation level for contraction, but would reduce the required increase in $[Ca^{2+}]_i$ for contraction.

Conclusions

In the mouse the response of contraction to increased frequency of stimulation is a fine balance between positive factors and negative factors, and the frequency response may thus be very sensitive to small changes in any of these factors. This may explain the existence of variability between cells, or between different mouse strains. Indeed, some authors have described a frank negative staircase (Wolska & Solaro, 1996; Ashley *et al.* 2001). The increase in baseline $[Ca^{2+}]_i$ at higher frequencies is likely to be important in the regulation of the frequency response. Higher basal $[Ca^{2+}]_i$ may be involved in the loss of $I_{Ca,L}$, but also in the increased contractile response, and perhaps in increased SERCA activity by promoting Ca^{2+}-dependent phosphorylation.

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