Modulation of focal and global Ca^{2+} release in calsequestrin-overexpressing mouse cardiomyocytes

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1. Focal and global Ca^{2+} releases were monitored in voltage-clamped control and hypertrophied calsequestrin (CSQ)-overexpressing mouse cardiomyocytes, dialysed with fluo-3, using rapid (120–240 frames s^{-1}) two-dimensional confocal imaging.

2. Spontaneous focal Ca^{2+} releases (Ca^{2+} sparks) were absent or significantly reduced in frequency in hypertrophied myocytes of CSQ-overexpressing mice compared to their age-matched controls. Sporadic Ca^{2+} sparks seen in CSQ-overexpressing myocytes had intensities and durations similar to those of controls although quantitative analysis showed a trend towards more diffuse focal releases.

3. Activation of Ca^{2+} current (I_{Ca}) failed to produce the typical sarcomeric Ca^{2+} striping pattern consistently seen in control myocytes. Instead, focal Ca^{2+} releases appeared as a disorganized patchwork of diffuse or 'woolly' fluorescence signals, resulting in slowly developing and reduced global Ca^{2+} transients.

4. Although the density of I_{Ca} in CSQ-overexpressing myocytes was only slightly smaller than that of controls, the inactivation kinetics of the current were greatly reduced, consistent with the much smaller rate of rise of cytosolic Ca^{2+}.

5. Enhancement of I_{Ca} by elevation of $[Ca^{2+}]_o$ from 2 to 10 mM or addition of 3 μM isoproterenol (isoprenaline) failed to normalize the frequency of spontaneous Ca^{2+} sparks at rest or the pattern and the magnitude of I_{Ca}-gated Ca^{2+} transients. Isoproterenol was somewhat more effective than elevation of $[Ca^{2+}]_o$.

6. In sharp contrast, low (0·5 mM) caffeine concentrations that produced no measurable effects on I_{Ca} or Ca^{2+} transients in control myocytes, re-established spontaneous focal Ca^{2+} releases in CSQ-overexpressing cells, triggered large I_{Ca}-gated cellular Ca^{2+} transients, and strongly enhanced the kinetics of inactivation of I_{Ca}.

7. Our data suggest that impaired Ca^{2+} signalling in CSQ-overexpressing myocytes results from reduced co-ordination and decreased frequency of Ca^{2+} sparks. The impaired Ca^{2+} signalling could not be restored by procedures that increased I_{Ca}, but was mostly restored in the presence of caffeine, which may alter the Ca^{2+} sensitivity of the ryanodine receptor.

In cardiac muscle the influx of Ca^{2+} through the Ca^{2+} channel is critical in triggering Ca^{2+} release from the sarcoplasmic reticulum (SR). The released Ca^{2+} in turn inactivates the L-type Ca^{2+} channel by interacting with the 'Ca^{2+} sensing' domains of the C-terminal tail (Soldatov *et al.* 1998) expressing calmodulin-binding properties (Zühlke *et al.* 1999; Peterson *et al.* 1999; Qin *et al.* 1999). Ca^{2+} mediated cross-signalling between the Ca^{2+} channel and ryanodine receptor (RyR) allows for tight functional coupling between these proteins, such that the influx of Ca^{2+} through the Ca^{2+} channel activates Ca^{2+} release from the RyR and the released Ca^{2+} in turn inactivates the Ca^{2+} channel, thereby inhibiting the release process (Sham *et al.* 1995). Quantitative analysis of the contribution of the released Ca^{2+} to the inactivation of the Ca^{2+} channel suggests that about 70 % of Ca^{2+} channel inactivation is determined by the Ca^{2+} released via the ryanodine receptor (Adachi-Akahane *et al.* 1996).

In the cardiac Ca^{2+} signalling cascade, the Ca^{2+} storage capacity of SR and the extent to which Ca^{2+} can be released from the low affinity ($K_d \approx 1$ mM) Ca^{2+} binding protein calsequestrin (CSQ) (MacLennan & Wong, 1971; Ikemoto *et al.* 1989) may also play a critical role in the release process (Kawasaki & Kasai, 1994). The structural properties of CSQ and its localization within the lumen of the SR suggest that this protein may interact with the RyR via the junctional proteins junctin and triadin (Jones, *et al.* 1995; Guo *et al.*

1996; Zhang *et al.* 1997). Recently we reported that mouse hearts overexpressing canine CSQ hypertrophied by 50–100% and developed heart failure (Jones *et al.* 1998). Isolated myocytes from such hearts were 50–100% larger and had significant electrophysiological and electromechanical alterations (Knollmann *et al.* 1998*b*). The most prominent features of electrical remodelling of these CSQ-overexpressing hearts were prolongation of the action potential and Q–T interval, downregulation of the transient outward K^+ current (I_{to}), and greatly slowed kinetics of inactivation of I_{Ca} (Jones *et al.* 1998; Knollmann *et al.* 1998*b*). Since I_{Ca}-gated Ca^{2+} release was impaired in CSQ-overexpressing myocytes despite the presence of 10- to 15-fold larger caffeine-releasable Ca^{2+} pools (Jones *et al.* 1998), we suggested that impaired Ca^{2+} release was mostly responsible for the marked slowing of the inactivation kinetics of I_{Ca}. In a subsequent report on another strain of transgenic mice overexpressing murine calsequestrin, similar hypertrophy and slowing of the kinetics of I_{Ca} were described, but the extent of impairment of I_{Ca}-gated Ca^{2+} release was not quantified (Sato *et al.* 1998).

In the present report we have quantified the properties of focal and global Ca^{2+} release in this model and have studied the nature of impaired Ca^{2+} signalling in CSQ-overexpressing myocytes by interventions that modify the Ca^{2+} channel or the Ca^{2+} sensitivity of the RyR. We find that agents that enhance Ca^{2+} current are less effective in re-establishing normal Ca^{2+} signalling than those that sensitize the Ca^{2+} release channel. A preliminary report of this work has already appeared (Wang & Morad, 1999).

METHODS

Transgenic mice and cell isolation

Mice expressing 10- to 15-fold higher levels of cardiac calsequestrin, aged 9–21 weeks, and showing significant cardiac hypertrophy were used in this study (Jones *et al.* 1998). Single ventricular myocytes were enzymatically isolated using a modification of a previously described collagenase–protease technique (Mitra & Morad, 1985). Briefly, mice were deeply anaesthetized with sodium pentobarbital (50 mg kg^{-1}, I.P.), the chest cavity was opened and hearts were excised, resulting in exsanguination. Experiments were conducted in accordance with institutional and NIH established guidelines. The excised hearts were placed in ice-cold incubation solution of the following composition (mM): 30 taurine, 90 NaCl, 5·4 KCl, 10 Hepes, 10 glucose and 1 $MgCl_2$, titrated to pH 7·2 with NaOH. The aorta was cannulated and perfused for 5 min with an oxygenated incubation solution at 36 °C containing 0·5 mM EGTA. The solution was then switched to an isolation solution containing 1 mg ml^{-1} albumin (Sigma Chemical Co.), 0·12 mg ml^{-1} protease (Type XIV, Sigma Chemical Co.) and 0·33 mg ml^{-1} collagenase (Type IV, 204 U mg^{-1}, Worthington Biochemical Corp.) for 8 min. The heart was then removed and the ventricle minced and digested for an additional one to three digestion periods (5–10 min each) in a shaking water bath at 37 °C. The resulting cell suspensions were collected after each digestion and stored at room temperature in an incubation solution containing an additional 0·2 mM $CaCl_2$. This procedure yielded 30–40% rod-shaped myocytes that were used for up to 10 h.

Electrophysiological measurements

Myocytes were whole-cell clamped (Hamill *et al.* 1981) using borosilicate glass capillaries (resistance 2–3 MΩ) filled with an internal Cs^+-rich solution (see below) containing 1 mM EGTA and 1 mM fluo-3. The cell capacitance was measured using previously established protocols (Cleemann & Morad, 1991). Currents were neither leak nor capacitance substracted (to obtain high quality data and to control for myocyte viability), but were series resistance compensated. Since imaging of the myocytes with a two-dimensional laser scanner required 10–15 min of dye dialysis, only cells with low leak current and clear striations were included in the final analysis of the results. All myocytes were clamped at a holding potential (V_h) of −70 mV prior to application of test pulses. Ca^{2+} currents were activated with test depolarizations to 0 mV either from −70 mV or more rarely from −50 mV to further suppress the residual TTX-resistant Na^+ current.

Two-dimensional confocal Ca^{2+} imaging

Ca^{2+} currents and 2-D Ca^{2+} images of a whole-cell-clamped myocyte were simultaneously monitored in cells dialysed with 1 mM fluo-3 and 1 mM EGTA. This concentration of dye in combination with EGTA was chosen to limit the diffusion distance of Ca^{2+} and Ca^{2+}–dye complex to about 50 nm (Adachi-Akahane *et al.* 1996; Cleemann *et al.* 1998). The combined use of fluorescent and non-fluorescent Ca^{2+} buffers and a confocal apparatus capable of rapidly scanning the cellular image via an accousto-optically steered laser beam (Noran, Odyssey, Madison, WI, USA) made it possible to obtain high resolution images of the rise and fall of cytosolic Ca^{2+} at high spatial and temporal resolution: ∼0·5 μm and 120–240 frames s^{-1} (Cleemann *et al.* 1998). Briefly, the confocal apparatus was mounted on an inverted microscope (Zeiss, Axiovert 135 TV) with a water immersion objective lens (Zeiss, C-apochromat, ×40, NA 1·2). The 488 nm excitation beam was generated from an argon ion laser (Omnichrome, Chino, CA, USA). The confocal slit in the rapidly scanned direction was set at 50 μm (0·8 μm in the focal plane), and the fluorescent light emitted was measured with a high efficiency photomultiplier tube (Hamamatsu, Middlesex, NJ, USA). The data were acquired by a Silicon Graphics workstation computer (Indy, Unix operating system, Noran Instruments, Middleton, WI, USA) and stored temporarily in 100 Mbyte random access memory before being transferred to hard disk.

Fluorescence measurements were initiated 6–8 min after rupture of the membrane under the patch pipette. After this period of dialysis, the intracellular fluo-3 concentration was typically close to its equilibrium near the patch electrode, but continued to exhibit slight gradients towards the ends of the cell (Cleemann *et al.* 1997). Static images of Ca^{2+}-dependent fluorescence show the average fluorescence intensity (F_0) calculated from several frames (Figs 1*A* and *B* (arrows) and 7*A* (arrows) and *B*). Dynamic Ca^{2+} signals were illustrated by sequences of frames showing the change in fluorescence (ΔF) relative to the average fluorescence (F_0) measured either in the presence of scattered focal Ca^{2+} releases (Figs 1 and 7), or immediately before and after Ca^{2+} releases activated by I_{Ca} (Figs 4*A* and *B* and 5*A* and *B*). This method of normalization was used to show Ca^2 sparks as clearly as possible without resorting to the use of contrast enhancement. In another approach (Figs 3*A* and *B*, 6*A* and *B* and 8*A* and *B*) we relied, in part, on a colour scale to present total fluorescence (F) with a large dynamic range. Tracings of cellular Ca^{2+} transients are shown as the average fluorescence of each frame normalized relative to the average resting fluorescence (F/F_0) prior to the ionic or pharmacological interventions (Figs 3*F* and *G*, 4*E*, 5*E*, 6*E* and *F*, and 8*E*). This type of normalization was used to permit comparison of results from different cells, and allow detection of systematic changes in both cellular transients (ΔF) and

resting fluorescence (F_r) during the experiment (Figs 4*F*, 5*F* and 8*F*). Focal Ca^{2+} releases were identified, and followed in time, using a computerized algorithm (Figs 2 and 7). This algorithm (Cleemann *et al.* 1998) identified local fluorescence maxima by means of a centre-minus-surround detection kernel (inset in Fig. 2), which consists of pixels (0·207 μm spacing) approximating a central positively weighted disc (radius 0·8 μm) and a concentric negatively weighted ring (radius 1–1·5 μm). A new computer algorithm was developed in Visual Basic (Microsoft) to remove constraints of the previous program and allow routine measurements of the amplitude and size of Ca^{2+} sparks by fitting a Gaussian distribution to the local fluorescence intensity (Fig. 1*C*).

Solutions and data analysis

The solution used for cellular equilibration and formation of the gigaseal contained (mM): 137 NaCl, 5·4 KCl, 2·0 $CaCl_2$, 1 $MgCl_2$, 10 Hepes, buffered to pH 7·4 with NaOH. In experimental solutions, inward K^+ currents were suppressed by addition of Ba^{2+} (0·2 mM) and omission of extracellular K^+. Na^+ currents were mostly suppressed using 3–6 μM TTX, and holding potentials (V_h) of −70 to −50 mV. We used 200 μM cAMP in all experiments except those where cells were exposed to isoproterenol (3 μM). In some experiments, elevation of $[Ca^{2+}]_o$ was achieved by adding the salt without correcting for the increase in osmolarity. Isoproterenol was used at 3 μM and caffeine at 0·5–10 mM. Patch pipettes were filled

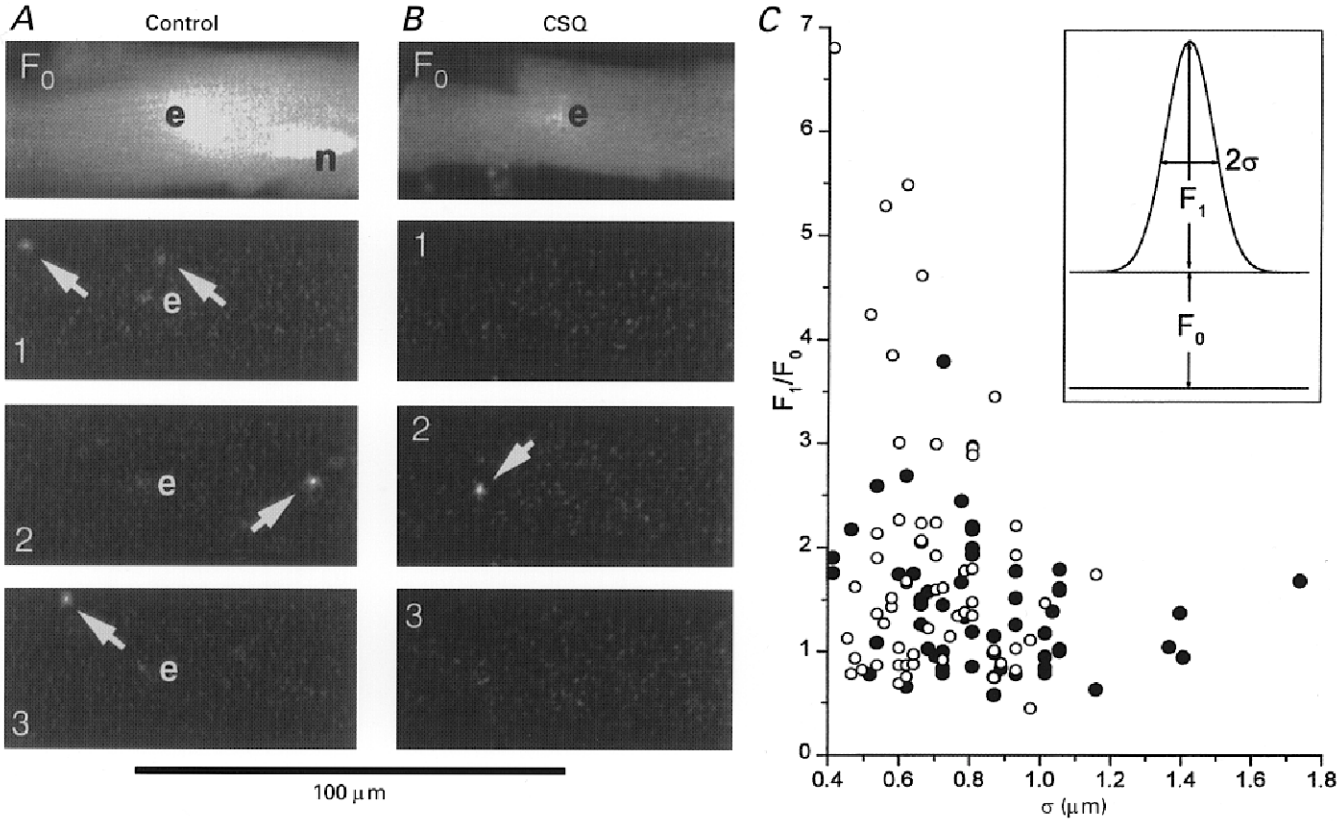

Figure 1. Spontaneous Ca^{2+} sparks in control (panel *A*) and CSQ-overexpressing (panel *B*) mouse cardiomyocytes and comparison of their size and magnitude (panel *C*)

The top images of two cardiomyocytes in panels *A* and *B* show the average fluorescence intensity (F_0) obtained from 60 frames recorded at 30 Hz in cells voltage clamped at a holding potential of −70 mV. In recordings obtained from 4 to 6 min after rupture of the patch, the fluorescence intensity is still highest near the dialysing patch electrode (e) suggesting that that the Ca^{2+}-sensitive dye (1 mM fluo-3 with 1 mM EGTA as an adjuvant) has not equilibrated fully in the longitudinal direction and/or that the dye in the pipette is partially detected even though it is outside the confocal plane. Nuclear regions (n) and faint longitudinal lines suggestive of fibrils are often seen. The lower images (1, 2 and 3) show three consecutive frames recorded at 33 ms interval in the two cells. The frames show the change in fluorescence (ΔF) so that the outline of the cell and the position of the electrode are seen only as changes in the intensity of the noise. Panel *A* shows the typical presence in control cells of one or more Ca^{2+} sparks in each frame. Panel *B* shows one of the rare Ca^{2+} sparks (arrow) in CSQ-overexpressing cells. Notice that the Ca^{2+} sparks are brief so that at 30 Hz they are seen clearly only in single frames and have, at most, a faint afterglow in the following frame (panel *B*, arrows). Panel *C* shows a scattergram with regression lines of the normalized intensity (F_1/F_0) and dimension (σ) of Ca^{2+} sparks in control (○) or CSQ-overexpressing (●) cells. The fluorescence intensity of each Ca^{2+} spark was fitted by least-squares approximation by a Gaussian distribution ($F_1\exp(-[(x-x_0)^2+(y-y_0)^2]/2\sigma^2)$) characterized by its centre (x_0, y_0), its standard deviation (σ, see inset) and the central increase in fluorescence (F_1) measured relative to the resting fluorescence (F_0).

with solutions containing (mM): 110 CsCl, 20 TEA, 10 Hepes, 5 MgATP, 5 glutathione, 1 EGTA, 1 fluo-3 (Molecular Probes Inc.), with the pH adjusted to 7·3 with CsOH. All experiments were performed at room temperature (22–25 °C).

Ensemble values were calculated as means ± S.E.M.

RESULTS

Transgenic mice overexpressing calsequestrin live into adulthood but develop significant cardiac dilatation and hypertrophy 30 days post partum (Knollmann *et al.* 1998*b*). Hypertrophy increases progressively with age, leading to heart failure. Isolated CSQ-overexpressing ventricular myocytes are also enlarged (~2-fold larger membrane capacitance; Jones *et al.* 1998) and have longer action potentials with significant downregulation of K^+ channels (I_{to} and I_{K1}; Knollmann *et al.* 1998*a*,*b*).

Spontaneous focal Ca^{2+} releases (Ca^{2+} sparks)

Figure 1 shows examples of two-dimensional confocal images of spontaneously occurring Ca^{2+} sparks in myocytes obtained from the hearts of CSQ-overexpressing mice (panel *B*) and their non-transgenic littermate (panel *A*). The two top images (F_0) show the average fluorescence intensity measured with the Ca^{2+} indicator dye fluo-3. The outline of each cell and the position of the whole-cell patch-clamp electrode (e) are clearly visible while the fading of the fluorescence signal towards the ends of the cells suggests that, following 6 min of dialysis, longitudinal equilibration of the cell with fluo-3 is still incomplete (see Cleemann *et al.* 1997). In Fig. 1*A* and *B* the lower three images (1, 2 and 3) are sequential frames at 30 Hz measured differentially as the increase in fluorescence (ΔF) relative to the average fluorescence (F_0). Consequently the outline of the cell and the position of the electrode are seen only as variations in the noise. On the other hand, the differential measurement aids the visual detection of Ca^{2+} sparks over the entire cell, including its edges. In control cells, spontaneous focal Ca^{2+} releases were almost always detected in normal Tyrode solution (2 mM $[Ca^{2+}]_o$, $V_h = -70$ mV), appeared well defined (0·8–2·4 μm in diameter corresponding to 2σ in panel *C*), and occurred randomly in different regions of the cells, typically at a rate of 1–2 per frame recorded at 30 Hz, covering the major part of the cell. In sharp contrast, spontaneously occurring Ca^{2+} sparks were absent in almost all transgenic myocytes, and in rare exceptions (7 out of 49 cells monitored for 1–2 s) they were generally seen only in a few singular frames (e.g. Fig. 1*B*).

Recordings at 30 frames s^{-1} were used to survey the major part of a cell typically for 2 s. In such records, each Ca^{2+} spark was seen clearly only in a single frame, and appeared, at best, as a much fainter diffuse fluorescence in the next frame consistent with a spark duration of less than 33 ms. These images were analysed by approximating individual Ca^{2+} sparks in single frames by a Gaussian distribution (inset of panel *C*) characterized by its normalized amplitude (F_1/F_0) and standard deviation (σ). Panel *C* compares the parameters of the sporadic spontaneous Ca^{2+} sparks measured in CSQ-overexpressing cells (●) to a randomly selected subset of the more numerous events in control cells (○). In terms of photons, the overall intensity of Ca^{2+} sparks (evaluated as the average of $\sigma^2 F_1/F_0$) was the same in control ($0{\cdot}91 \pm 0{\cdot}08\ \mu m^2$, $n = 60$ sparks) and CSQ-overexpressing cells ($1{\cdot}04 \pm 0{\cdot}10\ \mu m^2$, $n = 55$ sparks). Notice, however, that smaller ($\sigma = 0{\cdot}4$–$0{\cdot}8\ \mu m$) but very bright ($F_1/F_0 = 3$–7) Ca^{2+} sparks were only found in control cells (○) while very large sparks ($\sigma > 1{\cdot}2\ \mu m$) of lower intensity ($F_1/F_0 < 2$) appeared to be a characteristic of CSQ-overexpressing cells (●). This difference is based on a small number of observations, but it raises the possibility that Ca^{2+} sparks in CSQ-overexpressing cells tend to: (a) spread by diffusion during sustained Ca^{2+} release from a single site, (b) spread by Ca^{2+}-induced Ca^{2+} releases from a primary release site to some neighbouring sites, or (c) originate from more poorly defined or deformed diadic junctions (for ultrastructural deformity, see Jones *et al.* 1998).

To compare the time course of spontaneous Ca^{2+} sparks in control and CSQ-overexpressing myocytes the imaging speed was increased to 120 or 240 Hz, which reduced the scanned region (Fig. 2). In such experiments we were forced to choose the rare CSQ-overexpressing myocytes that produced a larger number of spontaneous diadic Ca^{2+} releases. Considering the variability in ultrastructural and electrophysiological properties of myocytes from CSQ-overexpressing hearts, we accepted the possibility that the more active CSQ-overexpressing myocytes were somewhat atypical of the fully developed stages of cardiac impairment. To obtain a valid comparison we therefore used for comparison control cells that also had a relatively high incidence of Ca^{2+} sparks and thus ascertained that the analysed CSQ-overexpressing myocytes were obtained from hearts with established hypertrophy and exhibited the characteristic slow kinetics of inactivation of I_{Ca} and Ca^{2+} transients (see Fig. 3). A computerized algorithm was used to locate the Ca^{2+} sparks and follow their development (Cleemann *et al.* 1998). The spatial resolution of these measurements was determined by a detection kernel, which measured the average fluorescence intensity over a disc with 0·8 μm radius relative to a surrounding ring of 1–1·5 μm radius. The selected traces of Fig. 2 suggest that Ca^{2+} sparks were of similar amplitude and duration in control (panels *A* and *C*, traces *a*–*m*) and in CSQ-overexpressing myocytes (panels *B* and *D*, traces *a*–*j*). The duration of focal Ca^{2+} release (15–20 ms) was somewhat briefer than that observed in most other studies (Cannell *et al.* 1994, 1995), probably because we have used both a centre-minus-surround kernel, and 1 mM EGTA as a secondary non-fluorescent Ca^{2+} buffer (for detailed analysis and rationale of this approach, see Cleemann *et al.* 1998). The 2-D confocal measurements made it possible to distinguish isolated focal Ca^{2+} releases from compound releases that spread slowly in the direction of the *z*-lines in control cells (Fig. 2*A* and *C*, arrows) and collectively lasted longer than the spatially confined releases. Such propagated releases were also seen occasionally in

CSQ-overexpressing cells (Fig. 2*B* and *D*, traces *e* and *f* and *h* and *i*), but we found no indication that this mechanism significantly enhances the overall level of spontaneously released Ca^{2+}. The cells illustrated in Fig. 2 had unusually high levels of spontaneous Ca^{2+} release, but the properties of each Ca^{2+} spark were similar to those typically observed in less active cells. When analysis was limited to Ca^{2+} sparks that showed no sign of propagation, and the analysis was performed with identical settings (detection threshold, dimensions of detection kernel, Cleemann *et al.* 1998), the average durations of sparks in control (17·3 ± 2·8 ms, $n = 4$ cells) and CSQ-overexpressing myocytes (17·6 ± 2·0 ms, $n = 8$ cells) were comparable.

Confocal images of I_{Ca}-gated Ca^{2+} release

Figure 3 compares the Ca^{2+} current and the profiles of rise of intracellular Ca^{2+} in a control and CSQ-overexpressing myocyte. Two-dimensional confocal images of Ca^{2+} obtained at 4·17 ms intervals (240 frames s^{-1}) shows that activation of I_{Ca} in the control myocyte leads to the development of sparks (arrows in panel *A*, frame 5; first filled circle in panel *F*) that appear as bright spots which tend to fuse into a sarcomeric Ca^{2+} striping pattern in later frames (Fig. 3*A*, frames 6 and 7; cf. Cleemann *et al.* 1998). This pattern of focal Ca^{2+} release generates rapidly activating and coordinated Ca^{2+} release (Fig. 3*F*). In CSQ-overexpressing myocytes, on the other hand, Ca^{2+} sparks do not develop into the characteristic sarcomeric Ca^{2+} stripes (Fig. 3*B*). Instead, large diffuse areas of Ca^{2+} fluorescence with embedded Ca^{2+} sparks (Fig. 3*B*, arrows) can be consistently observed during the slowly rising cytosolic Ca^{2+} transients (Fig. 3*G*). Note that the slow rise in cytosolic Ca^{2+} transients is accompanied by much slower inactivation of I_{Ca} (Fig. 3*D*), suggesting

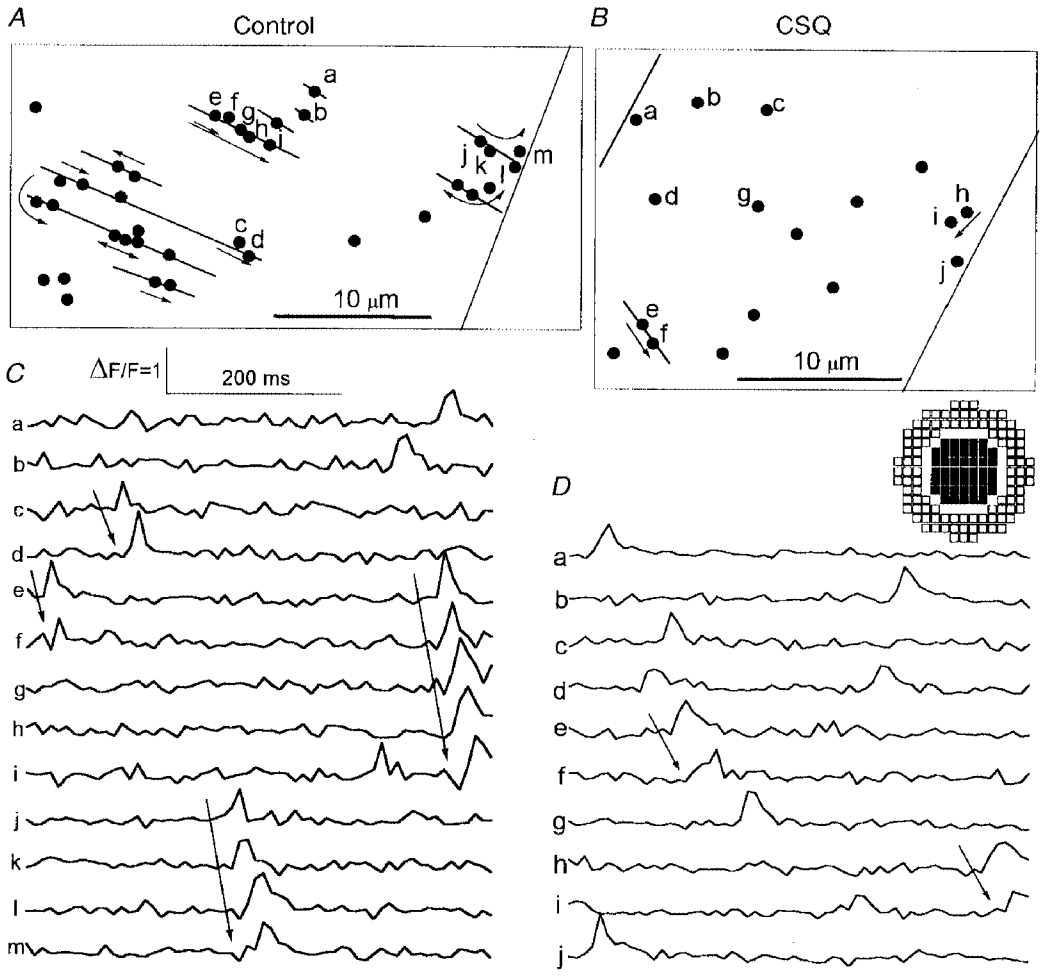

Figure 2. Time course and distribution of Ca^{2+} sparks in a control cell (panels *A* and *C*) and a CSQ-overexpressing cell (panels *B* and *D*)

Panels *A* and *B* show the location of all Ca^{2+} sparks (●) recorded in 60 frames at 120 Hz over a period of 500 ms in relation to the edges of the cell (nearly vertical lines) and the probable location of *z*-lines (panel *A*, nearly horizontal lines). Panels *C* and *D* illustrate the time course of Ca^{2+} sparks plotted as $\Delta F/F_0$, where ΔF is measured with a centre-minus-surround kernel (inset between panels *B* and *D*; see Methods), and F_0 is the average fluorescence intensity in the analysed area. In these abnormally active cells, what might appear as very intense, long lasting Ca^{2+} sparks could often be resolved as composites of several smaller Ca^{2+} sparks that were propagating primarily in the direction of the *z*-lines (→).

impaired Ca^{2+} signalling in CSQ-overexpressing myocytes (Adachi-Akahane *et al.* 1996; Jones *et al.* 1998).

The bar charts in Fig. 3 document the significance of these findings based on pooled data from a number of control and transgenic myocytes. Panel *H* compares the kinetics of Ca^{2+} transients and shows that the time-to-peak (TTP, see panel *F*) and the half-time ($t_{½}$) of its decay are much longer in CSQ-overexpressing myocytes, consistent with slower rise and fall of global cytosolic Ca^{2+}. Similarly, panel *E* shows that the half-time of inactivation of I_{Ca} ($t_{½}$, see Fig. 3*C*) in transgenic myocytes was prolonged more than 2-fold compared to the control myocytes. It is likely that the absence of coordinated and rapid Ca^{2+} release in CSQ-overexpressing myocytes is mostly responsible for the slower inactivation of I_{Ca} in transgenic mice, as has been described for Ca^{2+} currents in rat ventricular myocytes (Adachi-Akahane *et al.* 1996). In this analysis the half-time, rather than exponential analysis, was used to approximate the inactivation kinetics of the channel, as relatively brief 50–60 ms pulses were used in confocal imaging experiments to limit photo-damage caused by intense laser light.

Enhancement of I_{Ca} is not sufficient to restore Ca^{2+} signalling

Since the degree of activation of RyRs in part depends on a rapid rise of Ca^{2+} in the microenvironment of the Ca^{2+} channel–RyR complexes, we examined whether increasing the influx of Ca^{2+} through the channel would restore the impaired Ca^{2+} signalling in CSQ-overexpressing myocytes. To ensure that the Ca^{2+} content of the SR remained constant when 2 or 10 mM Ca^{2+} was used as charge carrier through the Ca^{2+} channel, a sequence of five conditioning depolarizing pulses in 2 mM Ca^{2+} were applied prior to the rapid and short (0·5 s) application of the 10 mM Ca^{2+} solution. Figure 4 shows the effect of elevation of extracellular Ca^{2+} from 2 to 10 mM on I_{Ca} (panels *C* and *D*), global cellular Ca^{2+} transients (panel *E* and *F*) and focal Ca^{2+} releases (panels *A* and *B*). Elevation of Ca^{2+} enhanced I_{Ca} (panel *D*, $32 \pm 9\%$, mean $\pm$ S.E.M., $n = 12$), increased the cellular Ca^{2+} transients ($\Delta F/F_0$ in panel *F*, by $100 \pm 9\%$, mean $\pm$ S.E.M., $n = 12$), and decreased the half-time of inactivation of I_{Ca} ($t_{½}$, panel *D*, $28 \pm 7\%$, mean $\pm$ S.E.M., $n = 12$); but the sporadic pattern of focal Ca^{2+} releases and their size, duration and uncoordinated nature remained unchanged.

Figure 5 shows that isoproterenol was somewhat more effective than elevation of Ca^{2+} in restoring the coordinated Ca^{2+} release, whether monitored as confocal images (frames of panels *A* and *B*) or as global intracellular Ca^{2+} transients (panels *E* and *F*). In the presence of isoproterenol, cytosolic Ca^{2+} transients were increased more than 2-fold ($133 \pm 35\%$, $n = 9$) as the resting Ca^{2+} signal increased by $65 \pm 5\%$ (mean $\pm$ S.E.M., $n = 9$, F_r/F_0 in panel *F*). Isoproterenol did not reduce the time-to-peak of global Ca^{2+} transients (panel *E*) or accelerate the inactivation kinetics of I_{Ca} significantly (panel *D*), consistent with the idea that coordinated Ca^{2+} release was not fully re-established. The increase in the number of Ca^{2+} sparks detected shortly after activation of I_{Ca} (frames 7–9 of panels *A* and *B*) appeared to be roughly proportional to the increase in the cellular Ca^{2+} transients (panel *E*), and occurred without significant changes in the size of the individual Ca^{2+} sparks. In this context, note that strong, long lasting focal Ca^{2+} releases, of the type seen near the edge of the cell in the lower right corner in each frame of both panels *A* and *B* (arrows), were detected both in the absence and presence of isoproterenol, but were too rare to contribute significantly to the global Ca^{2+} transient.

Caffeine improves the efficacy of Ca^{2+} release in CSQ-overexpressing myocytes

It has been previously reported that caffeine-releasable Ca^{2+} stores in CSQ-overexpressing myocytes were markedly enhanced even though I_{Ca}-gated Ca^{2+} release was impaired (Jones *et al.* 1998). In the next series of experiments, we attempted to examine the effects of high and low concentrations of caffeine on direct or I_{Ca}-gated Ca^{2+} release in CSQ-overexpressing transgenic myocytes. Figure 6 compares the effect of a rapid and short application ('puff') of 10 mM caffeine in control and transgenic myocytes. In control cells dialysed with 1 mM fluo-3 and 1 mM EGTA, rapid application of 5–10 mM caffeine triggered a rapid release of Ca^{2+}, the time course of which decayed slowly in the presence of caffeine (panel *A*, frames 13–70; panel *E*). Note that the rise in global Ca^{2+} concentrations activated only a brief and small Na^+–Ca^{2+} exchange current ($I_{Na\text{-}Ca}$; Callewaert *et al.* 1989), in part because cytosolic Ca^{2+} is well buffered by 1 mM EGTA and 1 mM fluo-3 (panel *C*; see also Adachi-Akahane *et al.* 1996). In sharp contrast, in similarly Ca^{2+}-buffered CSQ-overexpressing myocytes, the caffeine-induced Ca^{2+} release was so large as not only to activate a large $I_{Na\text{-}Ca}$ (panel *D*), but also to fully saturate the fluorescent dye signal (panel *F*, dashed line). These findings are consistent with the global measurements of Ca^{2+} in CSQ-overexpressing myocytes previously reported using high concentrations of fura-2 as Ca^{2+} buffer (Jones *et al.* 1998). Confocal images recorded at 30 Hz show that the pattern of caffeine-induced Ca^{2+} release was noticeably different in control (panel *A*) and transgenic (panel *B*) myocytes. In control cells, local Ca^{2+} releases were initiated at many sites, first close to the surface of the cell (panel *A*, frames 13 and 15) then at its centre (frames 17–21), and were followed by a more homogeneous rise in cytosolic Ca^{2+} that faded rapidly (frames 23–27), except for the delayed response from the nuclear region (frames 27 and 70). In contrast, the caffeine-induced Ca^{2+} signal in transgenic cells was typically initiated at only a few sites near the cell surface (panel *B*, frames 15 and 17), and then appeared to spread as a wave with some focal releases ahead of the front to the entire cell (frames 19 and 21), saturating the detector (frames 23 and 27) and causing noticeable cell shortening (frame 70), often irreversibly. Thus, it appears that the large Ca^{2+} release triggered by caffeine in CSQ-overexpressing cells may activate propagating Ca^{2+} waves (cf. Cheng *et al.* 1996).

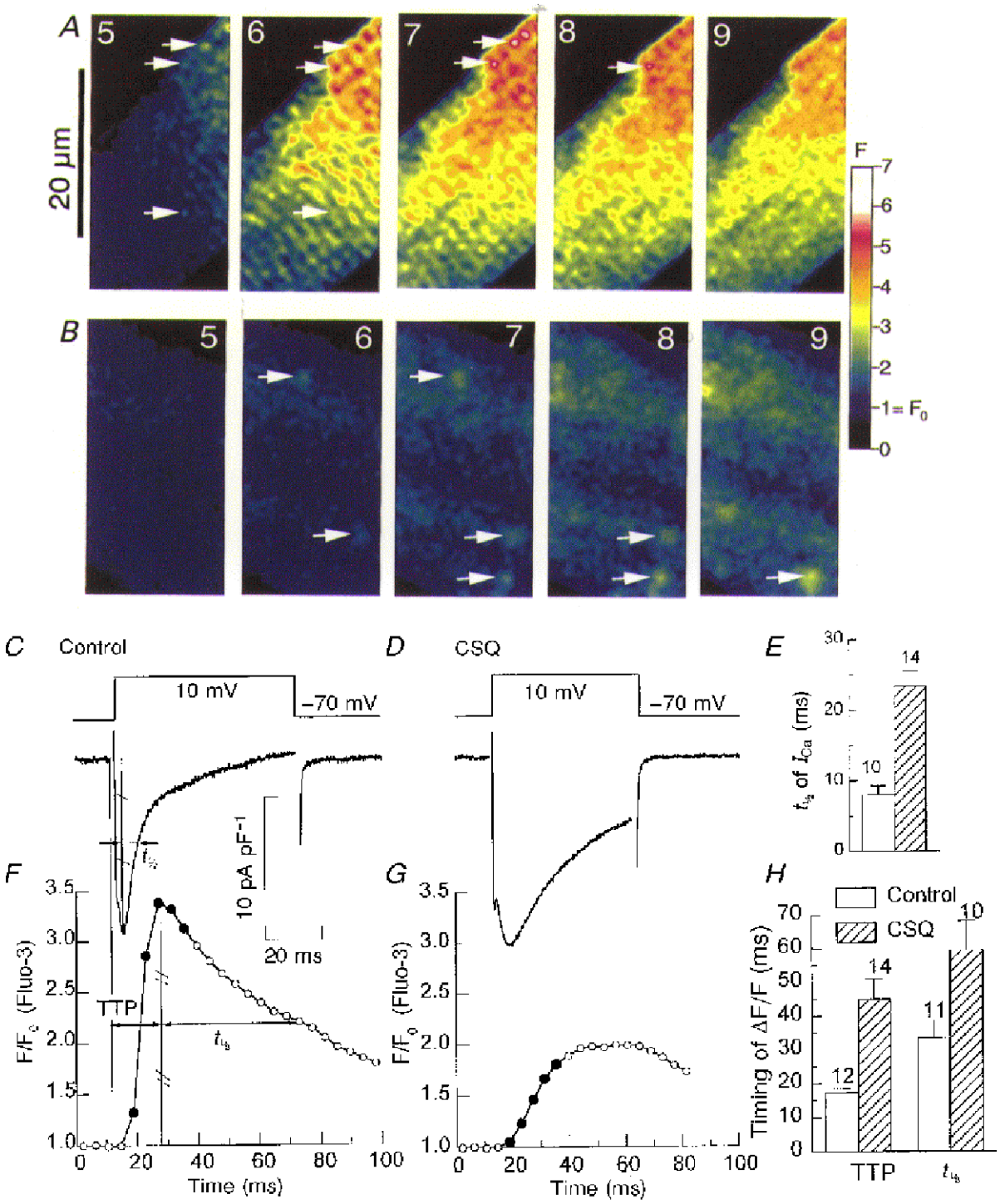

Figure 3. Comparison of I_{Ca}-induced Ca^{2+} release in voltage-clamped control (*A*, *C* and *F*) and transgenic (*B*, *D* and *G*) ventricular mouse cardiomyocytes

Panels *A* and *B* show numbered sample frames of total fluorescence (*F*) recorded at 240 Hz at the times indicated by filled symbols in the tracings of the cellular Ca^{2+} transients (panels *F* and *G*). Arrows identify some Ca^{2+} sparks that can be followed from frame to frame. The colour scale provides a calibration of the fluorescence intensity (*F*) relative to the resting fluorescence ($F_0 = 1$). Panels *C* and *D* illustrate the voltage-clamp protocol and the time course of the Ca^{2+} current. The bar charts compare the time course of the Ca^{2+} current (panel *E*) and the Ca^{2+} signal (panel *H*) in control (□) and CSQ-overexpressing (▨) cells. The Ca^{2+} current is characterized by its half-time of inactivation ($t_{½}$, see panel *C*) while the Ca^{2+} signal is described by both its time-to-peak (TTP, see panel *F*) and its half-time of decay ($t_{½}$). The error bars indicate the standard error of the mean and adjacent numbers indicate the number of cells examined.

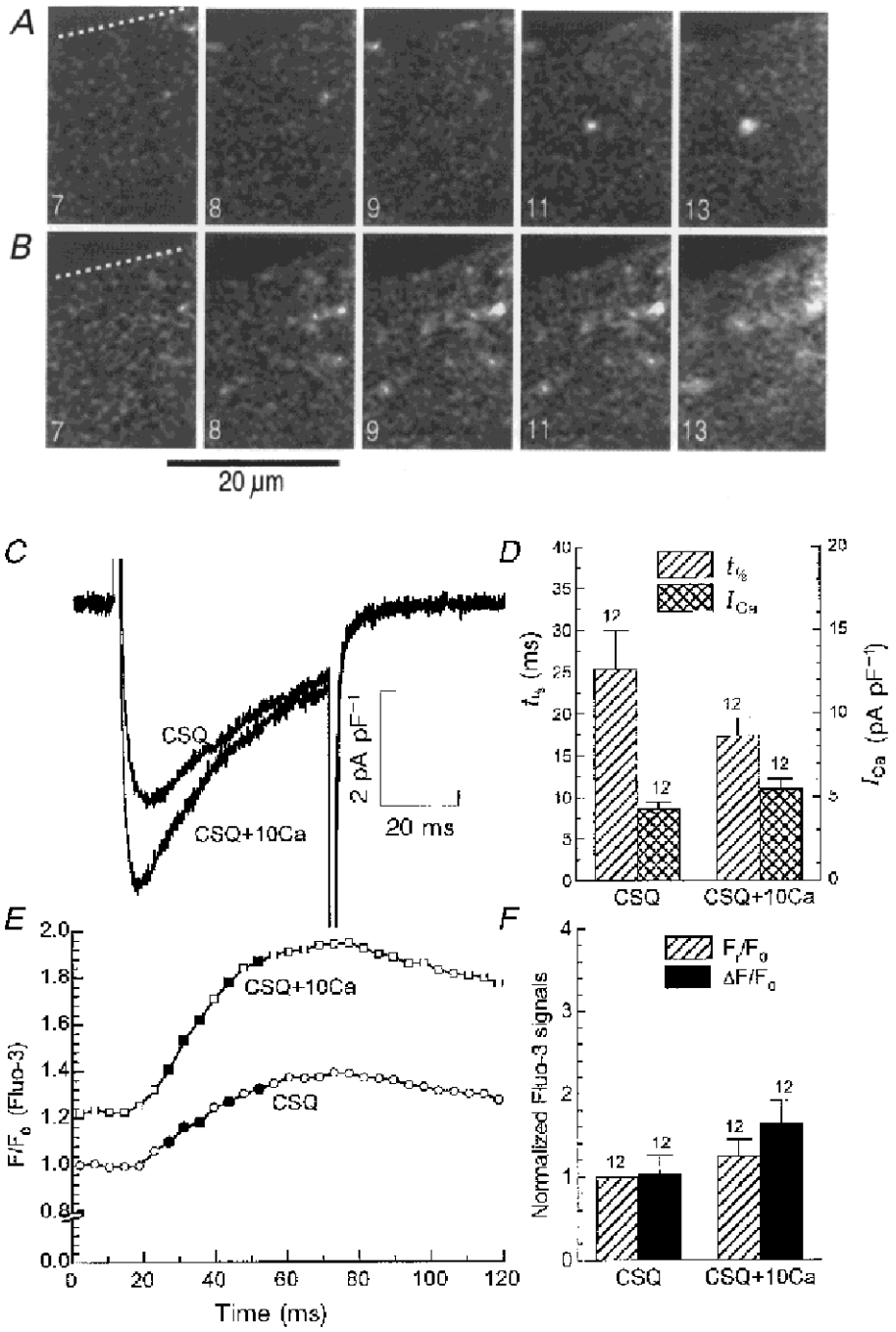

Figure 4. Effect of increased extracellular calcium concentrations on I_{Ca}-gated Ca^{2+} release in CSQ-overexpressing cells

Panels *A* and *B* show representative sample frames from stacks of fluorescence images (ΔF) recorded at 240 Hz during voltage-clamp depolarization from −70 to 0 mV with normal (2 mM, panel *A*) and elevated (10 mM, panel *B*) extracellular Ca^{2+} concentrations. Notice that elevation of $[Ca^{2+}]_o$ increased the number of Ca^{2+} sparks but did not change their appearance. The numbered sample frames (7, 8, 9, 11, 13 corresponding to filled symbols in panel *E*) show the increase in fluorescence (ΔF) after subtraction of the average background fluorescence. Panels *C* and *E* show, respectively, I_{Ca} and the cellular fluorescence signal in CSQ-overexpressing cardiomyocytes with 2 mM (CSQ) and 10 mM (CSQ+10Ca) $[Ca^{2+}]_o$. The fluorescence signals (*F*) in panel *E* were normalized relative to the resting cellular fluorescence (F_0) measured with 2 mM $[Ca^{2+}]_o$. Panel *D* shows average values of the amplitude of the current (I_{Ca}, right axis, ▨) and its half-time of inactivation ($t_{½}$, left axis, ▨) recorded with 2 mM (CSQ) and 10 mM (CSQ+10Ca) $[Ca^{2+}]_o$. Similarly, panel *F* shows the effect of elevated $[Ca^{2+}]_o$ on the average values of the resting (F_r, ▨) and transient (ΔF, ■) cellular fluorescence signals normalized relative to the resting fluorescence (F_0) measured with 2 mM Ca^{2+}. The numbers (12) next to the error bars indicate the number of cells examined.

To explore the effect of caffeine on spontaneous or Ca^{2+} channel-gated Ca^{2+} release in transgenic myocytes with intact Ca^{2+} stores, we tested the effects of lower concentrations of caffeine (0·5 mM) on focal and global Ca^{2+} releases. Figure 7*A* (frames 1 and 2) shows the spontaneous occurrence of a number of Ca^{2+} sparks in a CSQ-overexpressing myocyte clamped to −70 mV following 1 min exposure to 0·5 mM caffeine (cf. Fig. 1). This effect

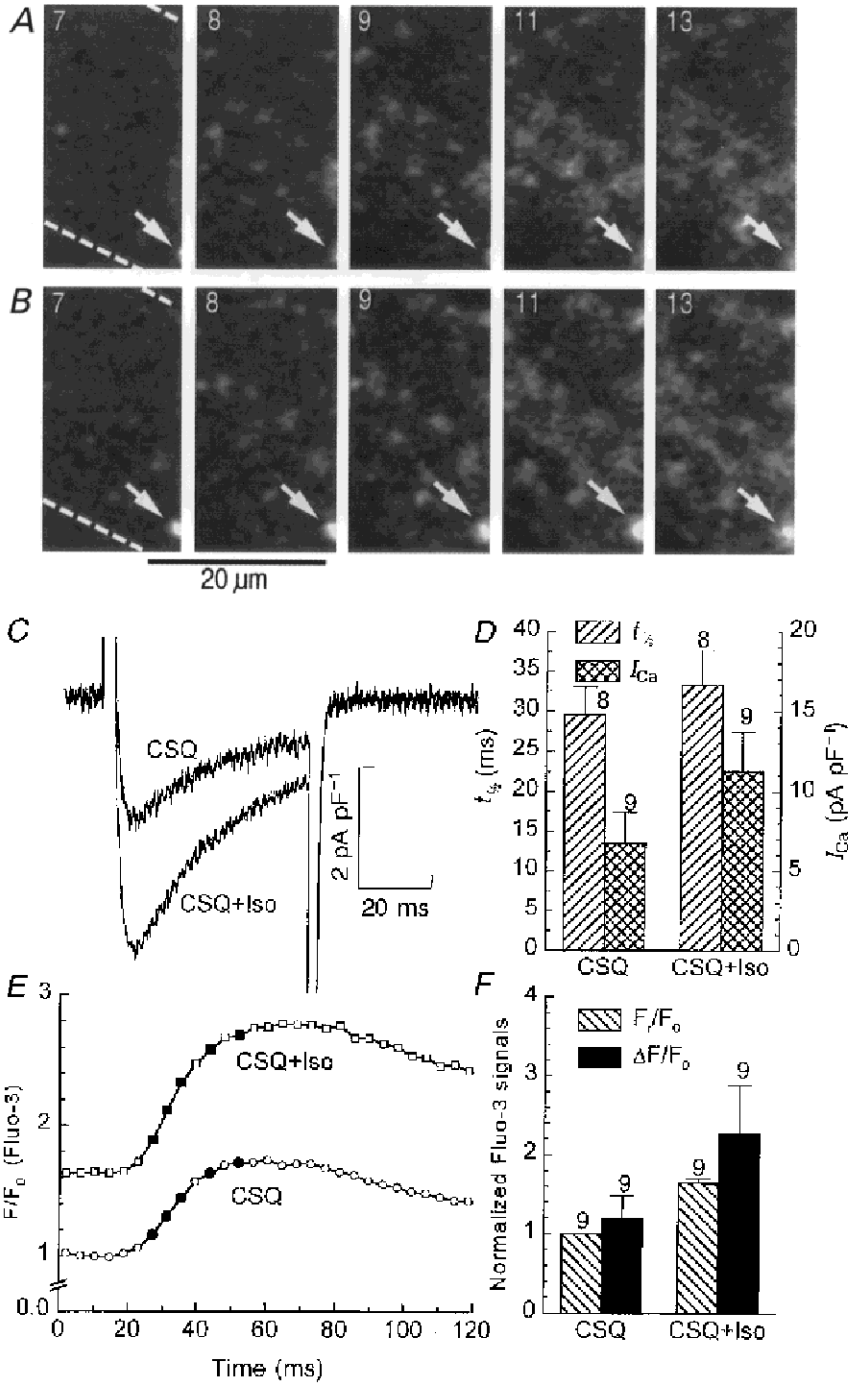

Figure 5. Effect of isoproterenol on I_{Ca}-gated Ca^{2+} release in transgenic cells

The layout of this figure is identical to that of Fig. 4. After the initial recording (CSQ), the transgenic cells were exposed to 3 μM isoproterenol for 1 min before testing the drug effect (CSQ+Iso). Panel *A*, sample frames before isoproterenol. Panel *B*, sample frames in the presence of isoproterenol. Arrows indicate the location of long lasting releases at a site that is barely visible in panel *A*, but in clearer view in panel *B* after some cell shortening. Panel *C*, membrane currents. Panel *D*, effect of isoproterenol on ensemble averages of half-time and amplitude of I_{Ca}. Panel *E*, Ca^{2+} transients. Panel *F*, effect of isoproterenol on ensemble averages of resting and transient fluorescence signals.

was observed in 20 out of 24 quiescent CSQ-overexpressing myocytes. In comparison, incubation of myocytes in 1 μM isoproterenol induced spontaneous Ca^{2+} sparks in 9 out of 13 quiescent CSQ-overexpressing myocytes. Figure 7 also shows the distribution (panel *B*) and time course of development of Ca^{2+} sparks in solution containing 0·5 mM caffeine (panel *C*, traces *a*–*i*). These focal Ca^{2+} releases occurred for the duration of caffeine exposure (seconds to minutes), were

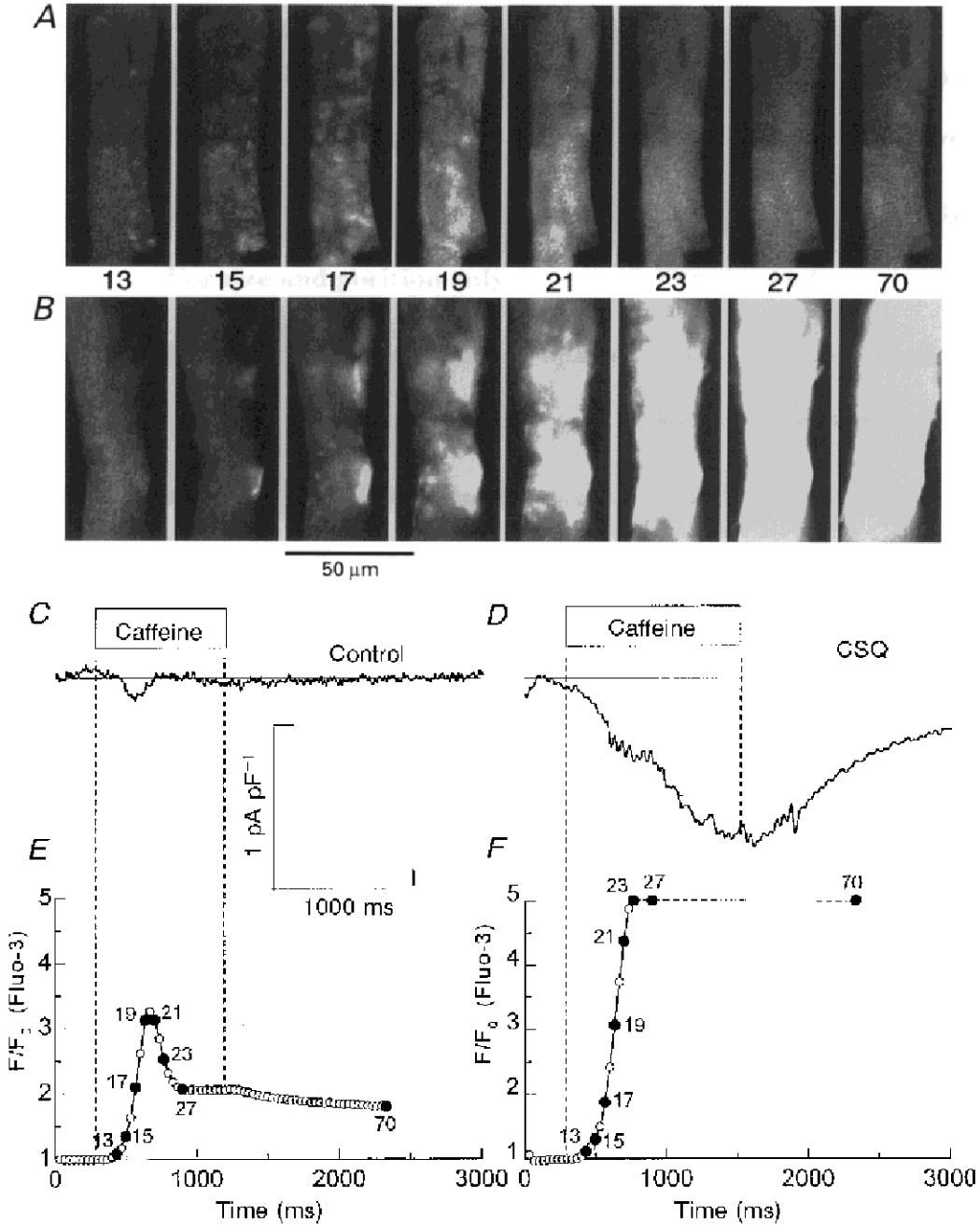

Figure 6. Caffeine-induced Ca^{2+} release in control (panels *A*, *C* and *E*) and transgenic (panels *B*, *D* and *F*) cardiomyocytes

The numbered sample frames from a control cell (panel *A*) and a transgenic cell (panel *B*) were recorded as total fluorescence (*F*) at 30 Hz at the times indicated by filled and numbered symbols in the fluorescence tracings (F/F_0) of panels *E* and *F*. The timing of the exposure to 10 mM caffeine is indicated by vertical dashed lines and the labelled bars (Caffeine) in panels *C* and *D*, which also show the time course of the Na^+–Ca^{2+} exchange current. Notice that frames 23 and 27 in panel *B* were recorded at a time when the inward Na^+–Ca^{2+} exchange current continued to increase, but the Ca^{2+} signal could no longer be followed because the fluorescence detector was in saturation (horizontal dashed line in panel *F*) and the Ca^{2+} waves were invading the ends of the cell outside the detection area.

markedly suppressed or absent upon washout of caffeine, and had intensities and durations similar to those seen in non-transgenic myocytes in the absence of caffeine (cf. Fig. 2).

Figure 8 compares I_{Ca}-gated Ca^{2+} transients and focal Ca^{2+} releases in the same transgenic myocyte in the absence and presence of 0·5 mM caffeine. Consistent with the findings of Fig. 3, depolarization of CSQ-overexpressing myocytes to 0 mV activated a slowly inactivating Ca^{2+} current (panel *C*, CSQ) which failed to release significant coordinated focal Ca^{2+} releases (panel *A*, frames 4–6) or global Ca^{2+} transients (panel *E*, CSQ). In the presence of 0·5 mM caffeine, however, global Ca^{2+} releases (panel *E*, CSQ + 0·5 mM Caff) recovered in response to activation of I_{Ca}. However, although some alignment in Ca^{2+} release sites was occasionally observed (dashed lines in panel *A*, frame 5 and panel *B*, frame 4), the characteristic Ca^{2+} striping pattern seen in control cells did not fully develop (panel *B*). The cellular response of CSQ-overexpressing myocytes in the presence of caffeine appeared to be composed of a large number of 'woolly' sparks, which were resolved most clearly shortly after depolarization or at the edges of the cells (panel *B*, arrows in frames 4 and 5), as they tended to fuse rapidly in the cell interior. It should be noted that the slow inactivation kinetics of I_{Ca} in CSQ-overexpressing myocytes ($t_{½}$, panel *D*)

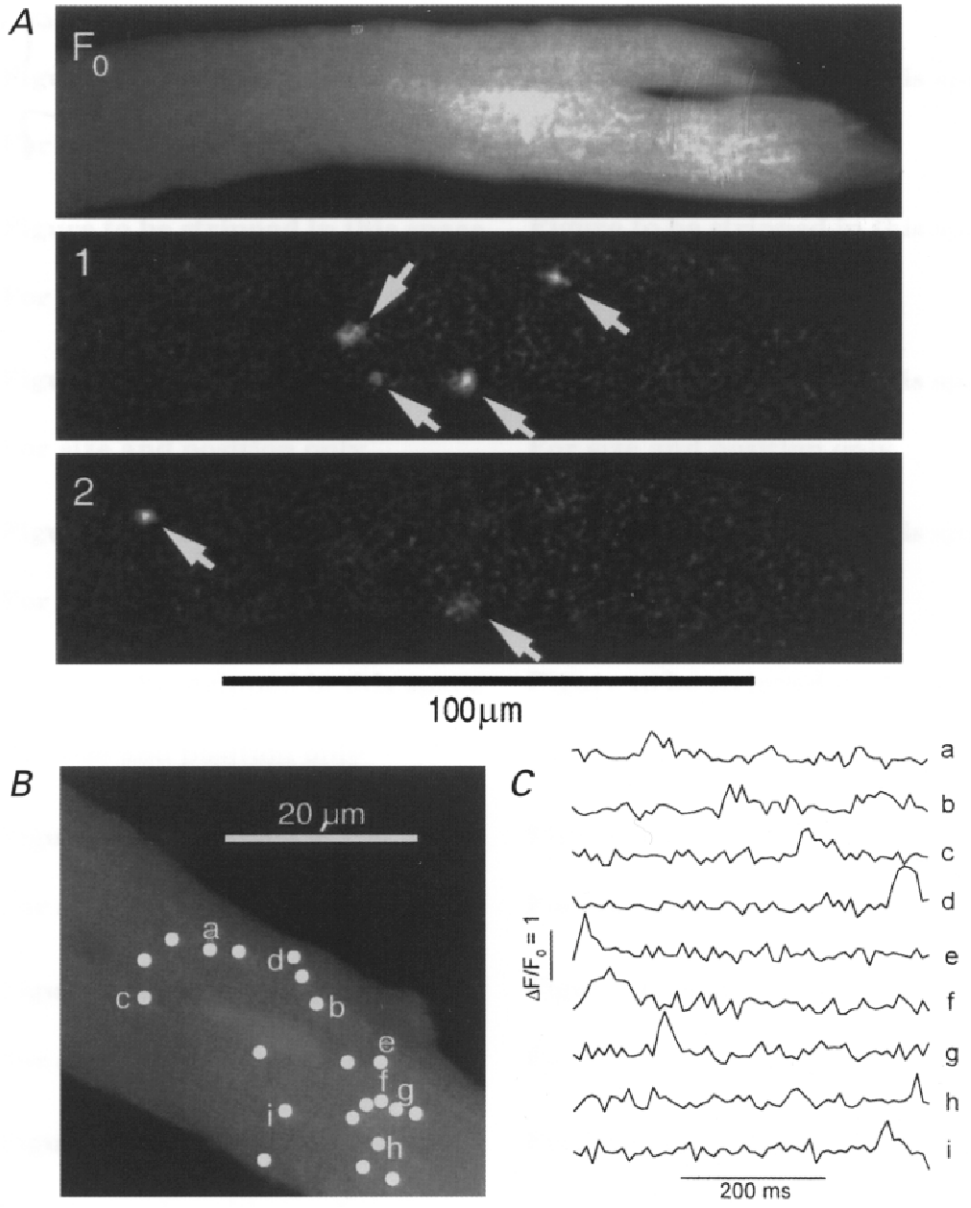

Figure 7. A low concentration of caffeine (0·5 mM) causes spontaneous Ca^{2+} spark activity in CSQ-overexpressing mouse cardiomyocytes

Panel *A* shows recordings in a layout similar to Fig. 1. Upper image, average fluorescence intensity (F_0). Lower images (1 and 2), sequential frames showing transient fluorescence changes (ΔF). Panel *B* shows the distribution and panel *C* the time course of Ca^{2+} sparks in a single cell exposed to 0·5 mM caffeine. The sample traces and their locations are labelled *a*–*i*.

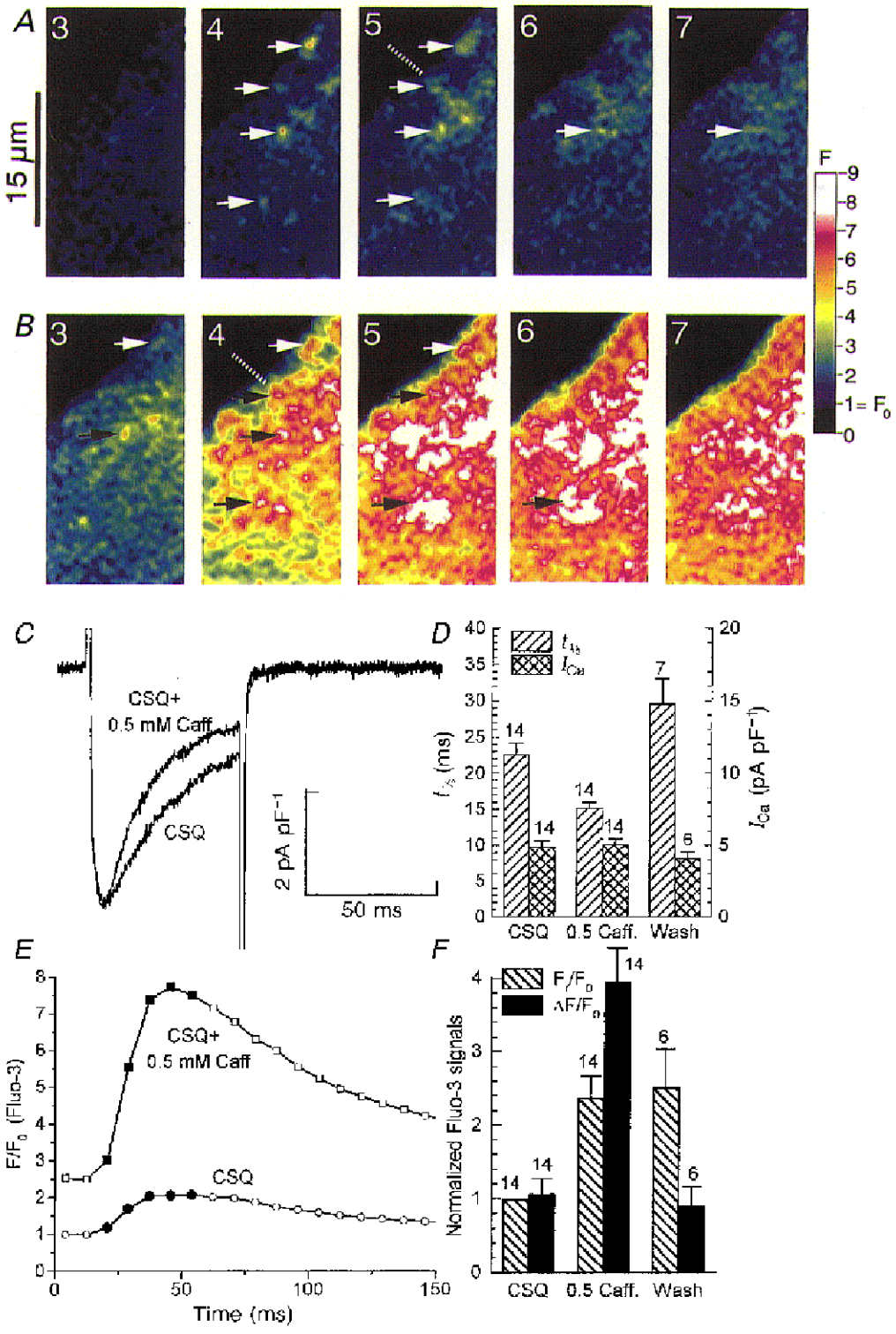

Figure 8. Caffeine in low concentrations strongly enhances the I_{Ca}-gated Ca^{2+} release in CSQ-overexpressing mouse cardiomyocytes

The sample frames of total fluorescence (F) were recorded at 120 Hz before (CSQ, panel A) and during (CSQ + 0·5 mM Caff, panel B) exposure to 0·5 mM caffeine during activation of I_{Ca} (panel C) at the times indicated by filled symbols in the tracings of the cellular fluorescence signals (panel E). Arrows in panels A and B show locations of prominent Ca^{2+} release sites; the dashed lines supposedly indicate alignment of release sites along a z-line; and the colour scale provides the means of calibration. In addition to the caffeine effect (0·5 mM Caff.) the ensemble averages in the bar graphs (panels D and F) also show the effect of washout (Wash).

were also significantly enhanced in the presence of 0·5 mM caffeine (panel *C*), consistent with the restoration of more effective Ca^{2+} release. Caffeine, however, had little or no effect on the peak magnitude of the Ca^{2+} current (Fig. 8*D*). The 3- to 4-fold enhancement of the cellular Ca^{2+} release induced by 0·5 mM caffeine was completely reversible ($\Delta F/F_0$, panel *F*) after washout of caffeine. The smaller increase in resting Ca^{2+} signal observed in the presence of caffeine, however, was not reversible (F_r/F_0, panel *F*), suggesting that photoinactivation, in part, contributes to the rise in the background signal.

In a few cells, where we used 1 and 2 mM concentrations of caffeine, we found that 1·0 mM caffeine consistently increased I_{Ca} and similarly enhanced the global Ca^{2+} transients. Caffeine at a concentration of 2 mM or higher, on the other hand, often induced partial Ca^{2+} release with transient suppression and then steady-state potentiation of I_{Ca} (data not shown).

DISCUSSION

The present data indicate that the impairment of Ca^{2+} release in myocytes overexpressing cardiac CSQ is associated with a reduced frequency of spontaneous or Ca^{2+} channel-gated Ca^{2+} sparks and the disappearance of the coordinated Ca^{2+} release which generates the sarcomeric Ca^{2+} striping pattern in non-transgenic cardiomyocytes. Effective Ca^{2+} signalling could be restored by low (0·5 mM) concentrations of caffeine, but not by agents that enhance I_{Ca} (isoproterenol and high $[Ca^{2+}]_o$), suggesting that impairment of I_{Ca}-gated Ca^{2+} release in CSQ-overexpressing myocytes may result from decreased Ca^{2+} sensitivity of the ryanodine receptors or their number in the diadic junctions.

Impaired Ca^{2+} release in CSQ-overexpressing transgenic myocytes

Although the caffeine-releasable Ca^{2+} pools in CSQ-overexpressing myocytes were enhanced (Fig. 6) 10- to 15-fold (Jones *et al.* 1998), the Ca^{2+} channel-gated Ca^{2+} release was small and uncoordinated (Fig. 3). We considered the possibilities that this reduction might occur because the unitary events associated with diadic Ca^{2+} release were either: (a) smaller in amplitude, (b) smaller in dimensions, or (c) fewer in number. To distinguish between these possibilities we studied spontaneous Ca^{2+} releases in control solutions and found that they occurred rarely in transgenic myocytes (Fig. 1*A* and B), but when they did sporadically occur, had the same magnitude as those of control cells (Fig. 1*C*). We cannot exclude the possibility that the release of Ca^{2+} in CSQ-overexpressing myocytes is distributed over a somewhat larger area (Fig. 1*C*), but found little evidence that this might result from the prolongation of the release process and the subsequent dispersion by diffusion. The measured duration (15–20 ms) of focal Ca^{2+} release in control and transgenic myocytes was somewhat longer than the value obtained by similar 2-D confocal measurements in rat ventricular myocytes (Cleemann *et al.* 1998), but was considerably shorter than line-scan estimates of the duration of Ca^{2+} sparks (Cheng *et al.* 1993; Cannell *et al.* 1994, 1995; Lopez-Lopez *et al.* 1995). It remains to be determined if this is due to differences in technique (target size (inset of Fig. 2), use of Ca^{2+} buffers, detection thresholds, scan mode, etc.) or reflects species-dependent variations.

Since large (5–10 mM) concentrations of caffeine produced propagated Ca^{2+} waves in CSQ-overexpressing cells, but not in control myocytes (Fig. 6*A* and *B*), we considered whether a similar mechanism might also contribute to the modulation of focal Ca^{2+} releases. We found that spontaneous Ca^{2+} releases sometimes showed saltatory propagation along *z*-lines, but this mechanism was not more pronounced in CSQ-overexpressing than in control myocytes (Fig. 2). It is more likely that propagated waves represent cells that are unusually active or Ca^{2+} overloaded.

The characteristic Ca^{2+} striping pattern of the I_{Ca}-activated Ca^{2+} release pattern in control cells (Fig. 3*A*) was absent (Figs 3*B*, 4 and 5) or only partially apparent (Fig. 8*A* and *B*, dashed lines) in transgenic myocytes. The analysis of spontaneous Ca^{2+} sparks and the patterns of Ca^{2+} release triggered by I_{Ca} indicate that the CSQ-overexpressing cells are capable of producing focal Ca^{2+} releases similar to those seen in control cells but with much lower frequency. This impairment may be, in part, related to downregulation of the expression of ryanodine receptors (Jones *et al.* 1998). In addition, ultrastructural distortion previously reported (Jones *et al.* 1998) may also alter the strategic location of RyRs, thereby diminishing their ability to sense influx of Ca^{2+} through the Ca^{2+} channel and trigger regenerative diadic Ca^{2+} releases. While transgenic cells show a spectrum of properties depending on the progression of hypertrophy with age (Knollmann *et al.* 1998*a*,*b*), it should be noted that the cells selected for detailed spark analysis came from hearts with established hypertrophy and characteristic slow Ca^{2+} kinetics of I_{Ca} and Ca^{2+} transients (Fig. 3).

Ca^{2+} channel activity in transgenic mice

The most prominent change in the biophysical properties of the Ca^{2+} channel in CSQ-overexpressing transgenic myocytes is its slow inactivation compared to control mice (Figs 3*D*, 4*C*, 5*C* and 8*C*) such that $t_{½}$ is increased from 8 to 24 ms (Fig. 3*E*). Such slow kinetics were first described by Jones *et al.* (1998) and were confirmed by Sato *et al.* (1998) in another murine CSQ-overexpressing transgenic strain. This effect appears to be unrelated to structural properties of the channel, as the kinetics of Ba^{2+} current through the Ca^{2+} channel of control and transgenic myocytes remained similar (data not shown). Furthermore, since the peak value of I_{Ca} shows little ($< 25\%$) or no change, the marked decrease in the rate of inactivation could not have resulted from a decrease in the influx of Ca^{2+} through the channel. We conclude therefore that the marked slowing of the kinetics of inactivation of I_{Ca} in CSQ-overexpressing myocytes of transgenic mice occurs secondary to the impaired Ca^{2+} release from the ryanodine receptor (Figs 3, 4, 5 and 8; see

also Jones *et al.* 1998). Such Ca^{2+} cross-talk has been quantified in rat ventricular myocytes where Ca^{2+} release appears to determine 60–70% of the Ca^{2+}-induced inactivation of the Ca^{2+} channel (Lee *et al.* 1985; Adachi-Akahane *et al.* 1996).

Recovery of I_{Ca}-gated Ca^{2+} release

Low doses of caffeine (0·5 mM) largely restored the ability of CSQ-overexpressing cells to produce both spontaneous Ca^{2+} sparks at rest (cf. Figs 1 and 7), and I_{Ca}-gated cellular Ca^{2+} transients (cf. Figs 3 and 8). In experiments with sufficient signal-to-noise ratio to resolve individual Ca^{2+} sparks, it was consistently observed that augmentation of cellular Ca^{2+} transients was accompanied, primarily, by an increased number of Ca^{2+} sparks (Figs 4 and 5) which, in the presence of 0·5 mM caffeine, tended to fuse together (Fig. 8). Individual Ca^{2+} sparks, under these conditions, were seen clearly only near the edges of the cell (Fig. 8). Considering also that the more clearly resolved spontaneous Ca^{2+} releases in transgenic cells are similar to those in control cells with respect to their amplitude, duration and confinement, it is likely that the major effect of caffeine is to increase the number of focal Ca^{2+} releases.

The large, almost 2- to 2·5-fold, increase in the myocyte surface area noted earlier (Jones *et al.* 1998) and confirmed here in CSQ-overexpressing myocytes may, in part, distort the ultrastructural organization of the DHP–ryanodine receptor complex making the Ca^{2+} release mechanism, which requires an organized dyadic microdomain, less efficient. This would especially be true if the level of expression of ryanodine receptor were depressed, as reported by Jones *et al.* (1998). In transgenic mice overexpressing murine cardiac calsequestrin, Sato *et al.* (1998) report no decrease in the level of cardiac ryanodine receptors or the associated junctional proteins junctin or triadin, but do report strongly modified kinetics of I_{Ca}, consistent with the compromised I_{Ca}-gated Ca^{2+} release. These authors did, however, find that the ryanodine-associated FKB12 protein was suppressed. Removal of FKB12 protein *in vitro* has been reported to be associated with development of multiple substate openings and 'chaotic' behaviour of the ryanodine receptor (Brillantes *et al.* 1994; Marks, 1996). Our 2-D confocal imaging, however, failed to confirm significant focal Ca^{2+} sparks at rest in CSQ-overexpressing myocytes (Fig. 1). Discrete sparks associated with the activation of Ca^{2+} channels were also rare and appeared to be replaced by fairly disorganized sparks which may be responsible for the slow and uncoordinated global intracellular Ca^{2+} rise in CSQ-overexpressing myocytes. Although ultrastructural studies might suggest distortions in dyadic junctions with SR (Jones *et al.* 1998), the finding that low concentrations of caffeine re-establish Ca^{2+} signalling is not quite consistent with major ultrastructural changes in the DHP–RyR complex or its immediate microenvironment; rather it may reflect increased sensitivity of ryanodine receptors to Ca^{2+} (Rousseau & Meissner, 1989; Sitsapesan & Williams, 1990). An alternative possibility for the impairment of Ca^{2+} signalling is that high levels of luminal Ca^{2+} may directly, or via the junctional protein, reduce the sensitivity of ryanodine receptor to Ca^{2+} or Ca^{2+} signalling via the Ca^{2+} channel. Thus, the impairment in Ca^{2+} signalling may be related primarily to functional interaction of Ca^{2+} channels with ryanodine receptors, rather than distortions in the ultrastructural architecture of the DHP–RyR complex.

Enhancement of I_{Ca} by elevation of $[Ca^{2+}]_o$ and isoproterenol was always less effective than low doses of caffeine in restoring I_{Ca}-gated Ca^{2+} release and producing only modest enhancements of the cellular Ca^{2+} transients (Figs 4*F* and 5*F*), which generally were related, but not strictly proportional, to changes in the magnitude and rate of inactivation of I_{Ca} (Figs 4*D* and 5*D*). Details of the observed responses may therefore reflect the fact that Ca^{2+} release is sensitive to changes in unitary Ca^{2+} current (Santana *et al.* 1996) and that inactivation of I_{Ca} is modulated not only by Ca^{2+} release, but also by screening charge effects of divalent cations, and modal shifts in the gating of I_{Ca} (McDonald *et al.* 1994). The variable effect of isoproterenol on recovery of Ca^{2+} signalling (data not shown) may reflect progression of the disease with age (Knollmann *et al.* 1998*a*) and the unexpected finding that enhancement of I_{Ca} by isoproterenol is pharmacologically different from that in control cells suggests a higher activity of phosphodiestrases in CSQ-overexpressing myocytes (Zhang *et al.* 1997; Knollmann *et al.* 1998*b*). Irrespective of these variations with age and experimental conditions, it seems unlikely that the β-adrenergic pathway can restore effective Ca^{2+} signalling in myocytes from CSQ-overexpressing mice with established cardiac hypertrophy.

The enhancements of the I_{Ca}-triggered Ca^{2+} releases by 10 mM $[Ca^{2+}]_o$, 3 μM isoproterenol, and, especially, 0·5 mM caffeine were paralleled at rest by changes in the basal Ca^{2+} signal (F_r/F_0, Figs 4*F*, 5*F* and 8*F*). This increase in resting signal failed to return fully to baseline after washout of these inotropic agents (e.g. caffeine, Fig. 8*F*), suggesting that: (1) the effect of caffeine on $[Ca]_i$ is long lasting, (2) resting cytosolic Ca^{2+} concentrations are gradually increasing (Cleemann & Morad, 1991), (3) dialysis of fluo-3 is still in progress at the time of measurement, or (4) photo-damaged fluo-3, though producing an increase in cellular fluorescence, is insensitive to Ca^{2+} (Lipp *et al.* 1996). It is likely that the apparent effect of caffeine on basal $[Ca^{2+}]_i$ (Fig. 8*E* and *F*) is real since it is much larger than the effects observed with elevation of $[Ca^{2+}]_o$ (Fig. 4*E* and *F*) and isoproterenol (Fig. 5*E* and *F*). To distinguish clearly between the different possibilities and examine the physiological consequences of this finding it would be useful to perform accurately calibrated ratiometric measurements using, for example, indo-1 or fura-2 as Ca^{2+} probes.

Animal models of cardiac hypertrophy and impaired Ca^{2+} signalling

The pathophysiology of impaired Ca^{2+} signalling, cardiac hypertrophy, and heart failure seen in CSQ-overexpressing mice is distinctly different from the phenotype associated

with other genetically modified animal models, including mice overexpressing the Na^+–Ca^{2+} exchanger, Ca^{2+}-ATPase, phospholamban, or the α_{1c} subunit of the Ca^{2+} channel, as well as spontaneously hypertensive rats, and salt-sensitive mice. Overexpression of the Na^+–Ca^{2+} exchanger (Adachi-Akahane *et al.* 1997; Terracciano *et al.* 1998), Ca^{2+}-ATPase (He *et al.* 1997; Yao *et al.* 1998), and phospholamban (Chu *et al.* 1997; Masaki *et al.* 1998), or the knockout of phospholamban (Masaki *et al.* 1997) produced no change in myocyte or heart size and did not impair Ca^{2+} signalling. The overexpression of the α_{1c} subunit of the Ca^{2+} channel produced a small (10–20 %) increase in cell size, but did not alter Ca^{2+} release (Muth *et al.* 1999). The overexpression of Ca^{2+}-ATPase or phospholamban and knockout of phospholamban appeared to regulate Ca^{2+} signalling only by altering the Ca^{2+} content of the SR. For instance, the increased SR Ca^{2+} content of phospholamban-deficient cardiomyocytes was reported to be directly related to an increased frequency of nearly unchanged Ca^{2+} sparks (Santana *et al.* 1997) and could be normalized by decreasing the Ca^{2+} content of the SR. Thus up- or downregulation of the Ca^{2+} content is not sufficient by itself to produce impairment of Ca^{2+} signalling or cardiac hypertrophy. In comparison, cells from hypertrophied hearts of spontaneously hypertensive rats produced larger Ca^{2+} sparks without a change in the SR Ca^{2+} content (Shorofsky *et al.* 1998).

Overexpression of CSQ and the α_{1c} subunit of the Ca^{2+} channel was unexpectedly accompanied by impaired β-adrenergic signalling (Zhang *et al.* 1997; Muth *et al.* 1999). Whether the defect in β-adrenergic signalling is a prerequisite for cardiac hypertrophy and failure, as previously suggested (Cho *et al.* 1999), remains to be determined. The impaired Ca^{2+} signalling of CSQ-overexpressing myocytes did not recover significantly in the presence of β-adrenergic agonists, as was reported for those of salt-sensitive mice in heart failure (Gomez *et al.* 1997). It might be of some interest to see if a modulation of Ca^{2+} signalling by caffeine is as effective in other models of heart failure as it is in murine CSQ-overexpressing myocytes. Although CSQ levels do not appear to change with cardiac disease in man, it is of interest that CSQ overexpression in mice exhibits the syndrome of hypertrophy and electrical remodelling almost identical to that found in human heart failure.

Adachi-Akahane, S., Cleemann, L. & Morad, M. (1996). Cross-signaling between L-type Ca^{2+} channels and ryanodine receptors in rat ventricular myocytes. *Journal of General Physiology* **108**, 435–454.

Adachi-Akahane, S., Lu, L., Li, Z., Frank, J. S., Philipson, K. D. & Morad, M. (1997). Calcium signaling in transgenic mice overexpressing cardiac Na^+-Ca^{2+} exchanger. *Journal of General Physiology* **109**, 717–729.

Brillantes, A., Ondrias, K., Scott, A. & Marks, A. (1994). Stabilization of calcium release channel (ryanodine receptor) by FK-506 binding protein. *Cell* **77**, 513–523.

Callewaert, G., Cleemann, L. & Morad, M. (1989). Caffeine-induced Ca^{2+} release activates Ca^{2+} extrusion via Na^+-Ca^{2+} exchanger in cardiac myocytes. *American Journal of Physiology* **268**, 1045–1049.

Cannell, M. B., Cheng, H. & Lederer, W. J. (1994). Spatial non-uniformities in $[Ca^{2+}]_i$ during excitation-contraction coupling in cardiac myocytes. *Biophysical Journal* **67**, 1942–1956.

Cannell, M. B., Cheng, H. & Lederer, W. J. (1995). The control of calcium release in heart muscle. *Science* **268**, 1045–1049.

Cheng, H., Lederer, M. R., Lederer, W. J. & Cannell, M. B. (1996). Ca^{2+} sparks and $[Ca^{2+}]_i$ waves in cardiac myocytes. *American Journal of Physiology* **270**, C148–159.

Cheng, H., Lederer, W. J. & Cannell, M. B. (1993). Calcium sparks: elementary events underlying excitation-contraction coupling in heart muscle. *Science* **262**, 740–744.

Cho, M.-C., Rapacciuolo, A., Koch, W. J., Kobayashi, Y., Jones, L. R. & Rockman, H. A. (1999). Defective β-adrenergic receptor signaling precedes the development of dilated cardiomyopathy in transgenic mice with calsequestrin overexpression. *Journal of Biological Chemistry* **274**, 22251–22256.

Chu, G., Dorn, G. W., Luo, W., Harrer, J. M., Kadambi, V. J., Walsh, R. A. & Kranias, E. G. (1997). Monomeric phospholamban overexpression in transgenic mouse heart. *Circulation Research* **81**, 485–492.

Cleemann, L., DiMassa, G. & Morad M. (1997). Ca^{2+} sparks within 200 nm of the sarcolemma of rat ventricular cells: Evidence form total internal reflection fluorescence microscopy. In *Analytical and Quantitative Cardiology*, ed. Siedman, S. & Beyer, R., pp. 57–65. Plenum Press, New York.

Cleemann, L. & Morad, M. (1991). Role of Ca^{2+} channel in cardiac excitation-contraction coupling in the rat: evidence from Ca^{2+} transients and contraction. *Journal of Physiology* **432**, 283–312.

Cleemann, L., Wang, W. & Morad, M. (1998). Two-dimensional confocal images of organization, density, and gating of focal Ca^{2+} release sites in rat cardiac myocytes. *Proceedings of the National Academy of Sciences of the USA* **95**, 10984–10989.

Gomez, A. M., Valdivia, H. H., Cheng, H., Lederer, M. R., Santana, L. F., Cannell, B. M., McCune, S. A., Altschuld, R. A. & Lederer, W. J. (1997). Defective excitation-contraction coupling in experimental cardiac hypertrophy and heart failure. *Science* **276**, 800–806.

Guo, W., Jorgenson, A. O., Jones, L. R. & Campbell, K. P. (1996). Biochemical characterization and molecular cloning of cardiac triadin. *Journal of Biological Chemistry* **271**, 458–465.

Hamill, O. P., Marty, A., Neher, E., Sakmann, B. & Sigworth, F. J. (1981). Improved patch-clamp technique for high resolution current recording from cells and cell-free patches. *Pflügers Archiv* **391**, 85–100.

He, H., Giordano, F. J., Hilal-Dandan, R., Choi, D.-J., Rockman, H. A., McDonough, P. M., Bluhm, W., Meyer, M., Sayen, R., Swanson, E. & Dillmann, W. H. (1997). Overexpression of the rat sarcoplasmic reticulum Ca^{2+} ATPase gene in the heart of transgenic mice accelerates calcium transients and cardiac relaxation. *Journal of Clinical Investigation* **100**, 380–389.

Ikemoto, N., Ronjat, M., Meszaros, L. G. & Koshita, M. (1989). Postulated role of calsequestrin in the regulation of calcium release from sarcoplasmic reticulum. *Biochemistry* **28**, 6764–6771.

Jones, L. R., Suzuki, Y. J., Wang, W., Kobayashi, Y. M., Ramesh, V., Franzini-Armstrong, C., Cleemann, L. & Morad, M. (1998). Regulation of Ca^{2+}-signaling in transgenic mouse cardiac myocytes overexpressing calsequestrin. *Journal of Clinical Investigation* **101**, 1385–1393.

Jones, L. R., Zhang, L., Sanborn, K., Jorgensen, A. O. & Kelley, J. (1995). Purification, primary structure, and immunological characterization of the 26-kDa calsequestrin binding protein (junctin) from cardiac junctional sarcoplasmic reticulum. *Journal of Biological Chemistry* **270**, 30787–30796.

Kawasaki, T. & Kasai, M. (1994). Regulation of calcium channel in sarcoplasmic reticulum by calsequestrin. *Biochemical and Biophysical Research Communications* **199**, 1120–1127.

Knollmann, B. C., Duc, J., Groth, A., Weissman, N. J., Cleemann, L. & Morad, M. (1998*a*). Cardiac phenotype of transgenic mice overexpressing calsequestrin. *Circulation* **98**, I-490.

Knollmann, B. C., Jones, L. R. & Morad, M. (1998*b*). Electrophysiologic properties of transgenic myocytes overexpressing cardiac calsequestrin. *Circulation* **98**, I-187.

Lee, K. S., Marban, E. & Tsien, R. W. (1985). Inactivation of calcium channel in mammalian heart cells: joint dependence on membrane potential and intracellular calcium. *Journal of Physiology* **364**, 395–411.

Lipp, P., Luscher, C. & Niggli, E. (1996). Photolysis of caged compounds characterized by ratiometric confocal microscopy: a new approach to homogeneously control and measure the calcium concentration in cardiac myocytes. *Cell Calcium* **19**, 255–266.

Lopez-Lopez, J. R., Shacklock, P. S., Balke, C. W. & Wier, W. G. (1995). Local calcium transients triggered by single L-type calcium currents in cardiac cells. *Science* **268**, 1042–1045.

McDonald, T. F., Pelzer, S., Trautwein, W. & Pelzer, D. J. (1994). Regulation and modulation of calcium channels in cardiac, skeletal and smooth muscle cells. *Physiological Reviews* **74**, 365–507.

MacLennan, D. H. & Wong, P. T. (1971). Isolation of a calcium-sequestering protein from sarcoplasmic reticulum. *Proceedings of the National Academy of Sciences of the USA* **68**, 1231–1235.

Marks, A. (1996). Molecular structure of calcium release channels. In *Molecular Physiology and Pharmacology of Cardiac Ion Channels and Transporters*, ed. Morad, M., Ebashi, S., Trautwein, W. & Kurachi, Y., pp. 397–408. Kluver Academic Publishers, Dordrecht, Netherlands.

Masaki, H., Sako, H., Kadambi, V. J., Sato, Y., Kranias, E. G. & Yatani, A. (1998). Overexpression of phospholamban alters inactivation kinetics of L-type Ca^{2+} channel currents in mouse atrial myocytes. *Journal of Cellular and Molecular Cardiology* **30**, 317–325.

Masaki, H., Sato, Y., Luo, W., Kranias, E. G. & Yatani, A. (1997). Phospholamban deficiency alters inactivation of L-type Ca^{2+} channels in mouse ventricular myocytes. *American Journal of Physiology* **272**, H606–612.

Mitra, R. & Morad, M. (1985). A uniform enzymatic method for dissociation of myocytes from hearts and stomachs of vertebrates. *American Journal of Physiology* **249**, H1056–1060.

Muth, J. N., Yamaguchi, H., Mikala, G., Grupp, I. L., Lewis, W., Cheng, H., Song, L.-S., Lakatta, E. G., Varadi, G. & Schwartz, A. (1999). Cardiac-specific overexpression of the α_1 subunit of the L-type voltage-dependent Ca^{2+} channel in transgenic mice. *Journal of Biological Chemistry* **274**, 21503–21506

Peterson, B. Z., DeMaria, C. D. & Yue, D. T. (1999). Calmodulin is the Ca^{2+} sensor for Ca^{2+}-dependent inactivation of L-type calcium channels. *Neuron* **22**, 549–558.

Qin, N., Olcese, R., Bransby, M., Lin, T. & Birnbaumer, L. (1999). Ca^{2+}-induced inhibition of the cardiac Ca^{2+} channel depends on calmodulin. *Proceedings of the National Academy of Sciences of the USA* **96**, 2435–2438.

Rousseau, E. & Meissner, G. (1989). Single cardiac sarcoplasmic reticulum Ca^{2+} release channel: activation by caffeine. *American Journal of Physiology* **256**, H328–333.

Santana, L. F., Cheng, H., Gomez, A. M., Cannell, M. B. & Lederer, W. J. (1996). Relation between the sarcolemmal Ca^{2+} current and Ca^{2+} sparks and local control theories for cardiac excitation-contraction coupling. *Circulation Research* **78**, 166–171.

Santana, L. F., Kranias, E. G. & Lederer, W. J. (1997). Calcium sparks and excitation–contraction coupling in phospholamban-deficient ventricular myocytes. *Journal of Physiology* **503**, 21–29.

Sato, Y., Ferguson, D. G., Sako, H., Dorn, G. W. II, Kadambi, V. J., Yatani, A., Hoit, B. D., Walsh, R. A. & Kranias, E. G. (1998). Cardiac-specific overexpression of mouse cardiac calsequestrin is associated with depressed cardiovascular function and hypertrophy in transgenic mice. *Journal of Biological Chemistry* **273**, 28470–28477.

Sham, J. S. K., Cleemann, L. & Morad, M. (1995). Functional coupling of Ca^{2+} channels and ryanodine receptors in cardiac myocytes. *Proceedings of the National Academy of Sciences of the USA* **92**, 121–125.

Shorofsky, S. R., Aggarwal, R., Corretti, M., Baffa J. M., Strum, J. M., Al-Seikhan, B. A., Kobayashi, Y. M., Jones, L. R., Wier, W. G. & Balke, C. W. (1998). Cellular mechanism of altered contractility in the hypertrophied heart. Big hearts, big sparks. *Circulation Research* **84**, 424–434.

Sitsapesan, R. & Williams, A. J. (1990). Mechanism of caffeine activation of single calcium-release channel of sheep cardiac sarcoplasmic reticulum. *Journal of Physiology* **423**, 425–439.

Soldatov, N. M., Oz, M., O'Brien, K. A., Abernethy, D. R. & Morad, M. (1998). Molecular determinants of L-type Ca^{2+} channel inactivation. *Journal of Biological Chemistry* **273**, 957–963.

Terracciano, C. M., De Souza, A. I., Philipson K. D. & MacLeod, K. T. (1998). Na^+-Ca^{2+} exchange and sarcoplasmic reticular Ca^{2+} regulation in ventricular myocytes from transgenic mice overexpressing the Na^+–Ca^{2+} exchanger. *Journal of Physiology* **512**, 651–667.

Wang, W. & Morad, M. (1999). Caffeine restores Ca signaling and spark formation in transgenic myocytes over-expressing calsequestrin. *Biophysical Journal* **76**, W-368.

Yao., A., Su, Z., Dillmann, W. & Barry, W. H. (1998). Sarcoplasmic reticulum function in murine ventricular myocytes overexpressing SR CaATPase. *Journal of Molecular and Cellular Cardiology* **30**, 2711–2718.

Zhang, L., Kelley, J., Schmeisser, G., Kobayashi, Y. M. & Jones, L. R. (1997). Complex formation between junctin, triadin, calsequestrin, and ryanodine receptor. Proteins of the cardiac junctional sarcoplasmic reticulum membrane. *Journal of Biological Chemistry* **272**, 23389–23397.

Zühlke, R. D., Pitt, G. S., Deisseroth, K., Tsien, R. W. & Reuter, H. (1999). Calmodulin supports both inactivation and facilitation of L-type calcium channels. *Nature* **399**, 159–162.

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