

# Regulation of Ca<sup>2+</sup> Signaling in Transgenic Mouse Cardiac Myocytes Overexpressing Calsequestrin

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## Abstract

To probe the physiological role of calsequestrin in excitation–contraction coupling, transgenic mice overexpressing cardiac calsequestrin were developed. Transgenic mice exhibited 10-fold higher levels of calsequestrin in myocardium and survived into adulthood, but had severe cardiac hypertrophy, with a twofold increase in heart mass and cell size. In whole cell–clamped transgenic myocytes, Ca<sup>2+</sup> channel-gated Ca<sup>2+</sup> release from the sarcoplasmic reticulum was strongly suppressed, the frequency of occurrence of spontaneous or Ca<sup>2+</sup> current–triggered “Ca<sup>2+</sup> sparks” was reduced, and the spark perimeter was less defined. In sharp contrast, caffeine-induced Ca<sup>2+</sup> transients and the resultant Na<sup>+</sup>-Ca<sup>2+</sup> exchanger currents were increased 10-fold in transgenic myocytes, directly implicating calsequestrin as the source of the contractile-dependent pool of Ca<sup>2+</sup>. Interestingly, the proteins involved in the Ca<sup>2+</sup>-release cascade (ryanodine receptor, junctin, and triadin) were downregulated, whereas Ca<sup>2+</sup>-uptake proteins (Ca<sup>2+</sup>-ATPase and phospholamban) were unchanged or slightly increased. The parallel increase in the pool of releasable Ca<sup>2+</sup> with overexpression of calsequestrin and subsequent impairment of physiological Ca<sup>2+</sup> release mechanism show for the first time that calsequestrin is both a storage and a regulatory protein in the cardiac muscle Ca<sup>2+</sup>-signaling cascade. Cardiac hypertrophy in these mice may provide a novel model to investigate the molecular determinants of heart failure. (*J. Clin. Invest.* 1998. 101:1385–1393.) Key words: calcium-induced calcium release • excitation–contraction coupling • hypertrophy • ryanodine receptor • sarcoplasmic reticulum

## Introduction

In the heart, Ca<sup>2+</sup> signaling is mediated by a sequence of events, starting with (a) opening of the L-type Ca<sup>2+</sup> channel

and activation of Ca<sup>2+</sup> current ( $I_{Ca}$ );<sup>1</sup> (b) Ca<sup>2+</sup> influx triggering the ryanodine receptors to release Ca<sup>2+</sup>; (c) the released Ca<sup>2+</sup> in turn inactivating the Ca<sup>2+</sup> channel, thus terminating the release process; (d) after the activation of myofilaments, the major fraction of released Ca<sup>2+</sup> (between 70 and 90%, depending on species) is sequestered by Ca<sup>2+</sup>-ATPase into the sarcoplasmic reticulum (SR), while another fraction is extruded from the cell via the Na<sup>+</sup>-Ca<sup>2+</sup> exchanger and sarcolemmal Ca<sup>2+</sup>-ATPase (1); and (e) Ca<sup>2+</sup> is stored in the SR by binding to calsequestrin before its release in the next cycle.

Cardiac calsequestrin binds 800–900 nmol Ca<sup>2+</sup> per mg of protein (~40 mol of Ca<sup>2+</sup>/mol of calsequestrin, with  $K_D = 0.4$ – $1$  mM at 150 mM KCl) (2), contains no transmembrane domains, and is located solely within the lumen of the SR (3). Structurally, calsequestrin contains 109 acidic amino acids with an excess of 69 negatively charged residues, contributing, in large part, to its ability to bind a large amount of Ca<sup>2+</sup> (3). Although the physiological role of calsequestrin is poorly understood, its association with terminal cisternae (4), its conformational change upon binding of Ca<sup>2+</sup> (2, 5), and its apparent anchoring to the ryanodine receptor via junctional face proteins such as triadin (6) and junctin (7, 8) suggest that calsequestrin may play a regulatory role in the Ca<sup>2+</sup>-release process (9–12).

To examine the physiological role of cardiac calsequestrin, transgenic mice with targeted overexpression of cardiac calsequestrin in atrium and ventricle were produced, and possible alterations in the Ca<sup>2+</sup>-signaling cascade were examined and quantified.

## Methods

**Generation of calsequestrin-overexpressing transgenic mice.** The 1378-bp SmaI restriction enzyme fragment from canine cardiac calsequestrin cDNA clone IC3A (3) was subcloned into pBluescript SK (Stratagene, La Jolla, CA) and then digested with Sall and SacI restriction enzymes. The Sall-SacI fragment, containing the entire canine cardiac calsequestrin protein coding region (including the signal peptide sequence) and 135 bp of 5'- and 10 bp of 3'-untranslated sequences, was ligated into the Sall-SacI sites of a mouse cardiac  $\alpha$ -myosin heavy chain ( $\alpha$ -MHC) promoter expression cassette (13). The expression cassette subcloned into pSPT included the  $\alpha$ -MHC promoter and 5'-untranslated region (a 5.7-kb insert described in Scott et al. [3]) fused to a 0.2-kb fragment containing the SV40 transcriptional terminator. The transgene, composed of the  $\alpha$ -MHC promoter, the entire protein coding region for calsequestrin, and the SV40 polyadenylation signal

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1. **Abbreviations used in this paper:** Ca<sub>i</sub>, intracellular Ca<sup>2+</sup>; [Ca<sup>2+</sup>]<sub>i</sub>, intracellular Ca<sup>2+</sup> concentration; I<sub>Ca</sub>, Ca<sup>2+</sup> current; I<sub>Na/Ca</sub>, Na<sup>+</sup>-Ca<sup>2+</sup> exchanger current;  $\alpha$ -MHC,  $\alpha$ -myosin heavy chain; SR, sarcoplasmic reticulum.

sequence was isolated from the parent plasmid as a 7.4-kb NruI fragment and used for embryo microinjections (14). Transgene-positive mice were identified by PCR assay of toe digests using primers designed to selectively amplify the transgene (15). Animals (6–9 wk old) were used in accordance with institutional guidelines.

**Immunoblotting.** Levels of SR proteins were determined by immunoblot analysis. 200  $\mu$ g (for detection of the ryanodine receptor) or 40  $\mu$ g (for detection of other SR proteins) of ventricular homogenate proteins was subjected to 5% (for ryanodine) or 8% (for other proteins) SDS-PAGE, blotted to nitrocellulose, and probed with antibodies specific for calsequestrin, ryanodine receptor, cardiac triadin isoform 1, junctin,  $\text{Ca}^{2+}$ -ATPase, and phospholamban (6, 7, 16). Blots were developed with  $^{125}\text{I}$ -protein A or alkaline phosphatase-conjugated protein A (for triadin). Immunoreactive bands were quantified by a molecular imager (model GS-250; Bio-Rad Laboratories, Richmond, CA).

**Electron microscopy.** Hearts from 4-wk-old wild-type and calsequestrin-overexpressing mice were fixed by perfusion through the left ventricle with a  $\text{Ca}^{2+}$ -free Ringer's solution, followed by 3.5% glutaraldehyde in 0.1 M cacodylate buffer. Small parallel bundles of fibers were teased from the walls of the left ventricle, postfixed in 2%  $\text{OsO}_4$  in 0.1 M cacodylate buffer, rinsed briefly, enblock stained with saturated uranyl acetate at 60°C for 4 h, and embedded in Epon. Sections were further stained with uranyl acetate and lead solutions and examined in an electron microscope (model EM410; Philips, Eindhoven, The Netherlands) (17).

**[ $^3\text{H}$ ]Ryanodine binding assay.** Homogenate protein (450  $\mu$ g) from control and MHC-calsequestrin mouse ventricles was added to 200  $\mu$ l of binding medium containing 20 mM Mops (pH 7.1), 1 mM  $\text{CaCl}_2$ , 0.6 M NaCl, and the saturating concentration of 15 nM [ $^3\text{H}$ ]ryanodine. Incubations were conducted at 37°C for 1 h in the presence and absence of 10  $\mu$ M cold ryanodine and terminated by filtration. Specific [ $^3\text{H}$ ]ryanodine binding is reported (7).

**Measurements of SR  $\text{Ca}^{2+}$  uptake.** 60 mg of mouse ventricular tissue from control and transgenic hearts were homogenized at 4°C in 1 ml of 0.25 M sucrose, 10 mM histidine (pH 7.4) with a homogenizer (model 2000; Omni International, Inc., Gainesville, VA). Active  $\text{Ca}^{2+}$  transport was determined at 37°C by adding 450  $\mu$ g of homogenate protein to 10 ml of  $\text{Ca}^{2+}$  uptake medium containing (in mM) 50 histidine, 3  $\text{MgCl}_2$ , 100 KCl, 5  $\text{NaN}_3$ , 3 oxalate, and 0.05  $^{45}\text{CaCl}_2$ . Samples were preincubated for 10 min in the presence or absence of 300  $\mu$ M ryanodine, and  $^{45}\text{Ca}^{2+}$  uptake was initiated by addition of 3 mM ATP and terminated at the indicated time points by filtration (Millipore Corp., Bedford, MA) (18, 19). 300  $\mu$ M ryanodine completely blocks open  $\text{Ca}^{2+}$ -release channels in junctional SR vesicles (18, 19). Oxalate precipitates actively sequestered  $\text{Ca}^{2+}$  retained inside SR vesicles, giving a 1,000-fold increase in uptake rates and negating any contribution by calsequestrin (18, 19).

**Cardiac myocyte isolation.** Ventricular myocytes were isolated from mice using the collagenase/protease method (20) that is a modification of the original method of Mitra and Morad (21). Briefly, animals were anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneal). Hearts were excised and perfused, at 1.2 ml/min in a Langendorff apparatus, first with  $\text{Ca}^{2+}$ -free Tyrode solution composed of (in mM) 137 NaCl, 5.4 KCl, 10 Hepes, 1  $\text{MgCl}_2$ , and 10 glucose (pH 7.3) at 37°C for 8 min, then with  $\text{Ca}^{2+}$ -free Tyrode solution containing collagenase (0.5 to 0.6 U/ml, type A; Boehringer Mannheim Biochemicals, Indianapolis, IN) and protease (0.55 U/ml, type XIV, pronase E; Sigma Chemical Co., St. Louis, MO) for 15 min, and finally with low  $\text{Na}^+$ , high sucrose Tyrode solution containing (in mM) 52.5 NaCl, 4.8 KCl, 1.19  $\text{KH}_2\text{PO}_4$ , 1.2  $\text{MgSO}_4$ , 11.1 glucose, 145 sucrose, 10 Hepes, 0.2  $\text{CaCl}_2$  for 10 min. The ventricle of the digested heart was then cut into several sections and subjected to gentle agitation to dissociate the cells. The freshly dissociated cells were stored at 25°C in low  $\text{Na}^+$ , high sucrose Tyrode solution containing 0.2 mM  $\text{CaCl}_2$  and were used for up to 10 h after isolation.

**Patch-clamp experiments.** The patch electrodes, made of borosilicate glass capillaries (resistance of 2–3 M $\Omega$ ) were filled with the in-

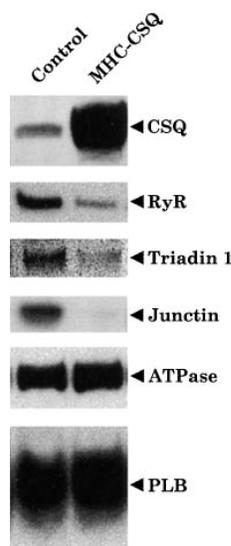
ternal solution composed of (in mM) 110 CsCl, 30 tetraethylammonium chloride, 5 MgATP, 10 Hepes, 7 NaCl, 0.2 cAMP, and  $\text{K}_5\text{fura-2}$  (Molecular Probes, Inc., Eugene, OR) and titrated to pH 7.2 with CsOH. Cells were perfused with Tyrode solution containing 2 mM  $\text{CaCl}_2$  and 0.2 mM  $\text{BaCl}_2$ . Whole cell-clamped myocytes were dialyzed with the internal solution for at least 8 min, and current and SR  $\text{Ca}^{2+}$  release transients were monitored simultaneously using an integrating patch amplifier (model 3900A; Dagan Corp., Minneapolis, MN) and a rapidly alternating (1.2 kHz) dual beam excitation fluorescence photometry set-up (Vibraspec Inc., Bear Island, ME) (22) at 25°C. Excitation wavelengths of 335 and 410 nm were used to monitor the fluorescence signals of  $\text{Ca}^{2+}$ -bound and  $\text{Ca}^{2+}$ -free fura-2 (22). The signals were acquired simultaneously with the whole cell currents using pCLAMP software, and intracellular concentration of  $\text{Ca}^{2+}$  ( $[\text{Ca}^{2+}]_i$ ) was calculated (23). Since rapid application of caffeine causes intracellular  $\text{Ca}^{2+}$  release and activates inward  $\text{Na}^+$ - $\text{Ca}^{2+}$  exchanger current ( $I_{\text{Na/Ca}}$ ) (24), caffeine-induced SR  $\text{Ca}^{2+}$  release and  $I_{\text{Na/Ca}}$  were monitored simultaneously at –90 mV. Cells were exposed to 5 mM caffeine for 1.8 s using a rapid concentration clamp system (22). The transient inward current ( $I_{\text{Na/Ca}}$ ) generated by caffeine-induced  $\text{Ca}^{2+}$  release represents exchange of three  $\text{Na}^+$  for one  $\text{Ca}^{2+}$ .

**Confocal imaging.**  $\text{Ca}^{2+}$  currents and two-dimensional  $\text{Ca}^{2+}$  images were monitored simultaneously in myocytes dialyzed with internal solutions containing fluo-3 (Molecular Probes, Inc.). Fluorescence images were recorded using an acoustooptically steered confocal microscope (Noran Instruments, Milton Keynes, UK) at 120 frames/s with a pixel size of 0.207  $\mu$ m and 3  $\times$  3 filtering before plotting on a common color scale.

**Statistical analysis.** Means  $\pm$  SEM were calculated, and statistically significant differences between two groups were determined by the Student's *t* test at *P* < 0.05.

## Results

**Overexpression of calsequestrin alters gene expression of  $\text{Ca}^{2+}$ -regulatory proteins and causes cardiac hypertrophy.** The level of the expression of calsequestrin in transgenic hearts was 10-fold higher than in control mice as quantified by Western blot analysis (Fig. 1). Remarkably, other proteins of the junctional SR, the ryanodine receptor, triadin, and junctin were downregulated by 50% or more, whereas the levels of proteins associated with active sequestration of  $\text{Ca}^{2+}$ ,  $\text{Ca}^{2+}$ -ATPase, and phospholamban were increased slightly (10–25% for  $\text{Ca}^{2+}$ -ATPase) or were unchanged (Fig. 1). Such mice, though living



**Figure 1.** Immunoblots detecting cardiac SR proteins in homogenates from control and MHC-calsequestrin mouse ventricles. 200  $\mu$ g (for detection of the ryanodine receptor) or 40  $\mu$ g (for detection of other SR proteins) of ventricular homogenate proteins was subjected to SDS-PAGE, blotted to nitrocellulose, and probed with antibodies specific for calsequestrin (CSQ), ryanodine receptor (RyR), cardiac triadin isoform 1 (Triadin I), junctin,  $\text{Ca}^{2+}$ -ATPase, and phospholamban (PLB). Blots were developed with  $^{125}\text{I}$ -protein A or alkaline phosphatase-conjugated protein A (triadin), and the corresponding autoradiogram is shown.

Table I. Cardiac Properties of Calsequestrin-overexpressing Transgenic Mice

|   | Control           | Calsequestrin        |
|---|-------------------|----------------------|
| Heart weight (mg)   | 85.7±3.6 (n = 28) | 162±8.5 (n = 25)*    |
| Body weight (g)   | 20.1±0.8 (n = 16) | 19.3±0.9 (n = 13)    |
| Heart weight/body weight ratio                                  | 4.3               | 8.4                  |
| Capacitance (pF)  | 151±6 (n = 23)    | 240±12 (n = 54)*     |
| Calcium current, $I_{Ca}$ (pA/pF)                               |                   |                      |
| (0.1 mM fura-2)   | 11.6±1.1 (n = 3)  | 7.8±0.9 (n = 8)      |
| (2 mM fura-2)   | 7.1±3.2 (n = 4)   | 7.3±2.0 (n = 6)      |
| (1 mM fluo-3, 1 mM EGTA)  | 9.8±1.8 (n = 5)   | 11.4±1.9 (n = 5)     |
| (1 mM fluo-3, 14 mM EGTA)                                       | 11.3±2.1 (n = 3)  | 10.1±3.8 (n = 3)     |
| Time constant of $I_{Ca}$ (ms)                                  |                   |                      |
| (0.1 mM fura-2)   | 7.3±1.4 (n = 3)   | 26.1±2.0 (n = 8)*    |
| (2 mM fura-2)   | 8.9±0.5 (n = 4)   | 35.7±1.4 (n = 5)*    |
| (1 mM fluo-3, 1 mM EGTA)  | 6.4±1.1 (n = 5)   | 21.4±3.9 (n = 5)*    |
| (1 mM fluo-3, 14 mM EGTA)                                       | 14.9±9.4 (n = 3)  | 33.1±7.8 (n = 2)     |
| Ca <sub>i</sub> -transient ( $I_{Ca}$ -induced)                 |                   |                      |
| (0.1 mM fura-2)   | 679±41 nM (n = 3) | 238±89 nM (n = 3)*   |
| (2 mM fura-2)   | 110±23 nM (n = 4) | 17.6±7.6 nM (n = 6)* |
| (1 mM fluo-3, 1 mM EGTA)  | 209±51% (n = 4)   | 106±39% (n = 6)      |
| (1 mM fluo-3, 14 mM EGTA)                                       | 84±23% (n = 3)    | 37±21% (n = 5)       |
| [ <sup>3</sup> H]Ryanodine binding (fmol/mg homogenate protein) | 377±28            | 207±13*              |

$I_{Ca}$  and Ca<sub>i</sub>-transients were measured in whole cell-clamped myocytes dialyzed with the indicated Ca<sup>2+</sup> buffers. Fura-2 measurements of Ca<sup>2+</sup> are calibrated (see citation to Fig. 3), whereas measurements with fluo-3 are percentage relative to resting fluorescence (see citation to Fig. 5). Holding potentials were -50 to -70 mV, and the duration of voltage clamp pulses was 60–100 ms. \*Significantly different from control values at  $P < 0.05$ .

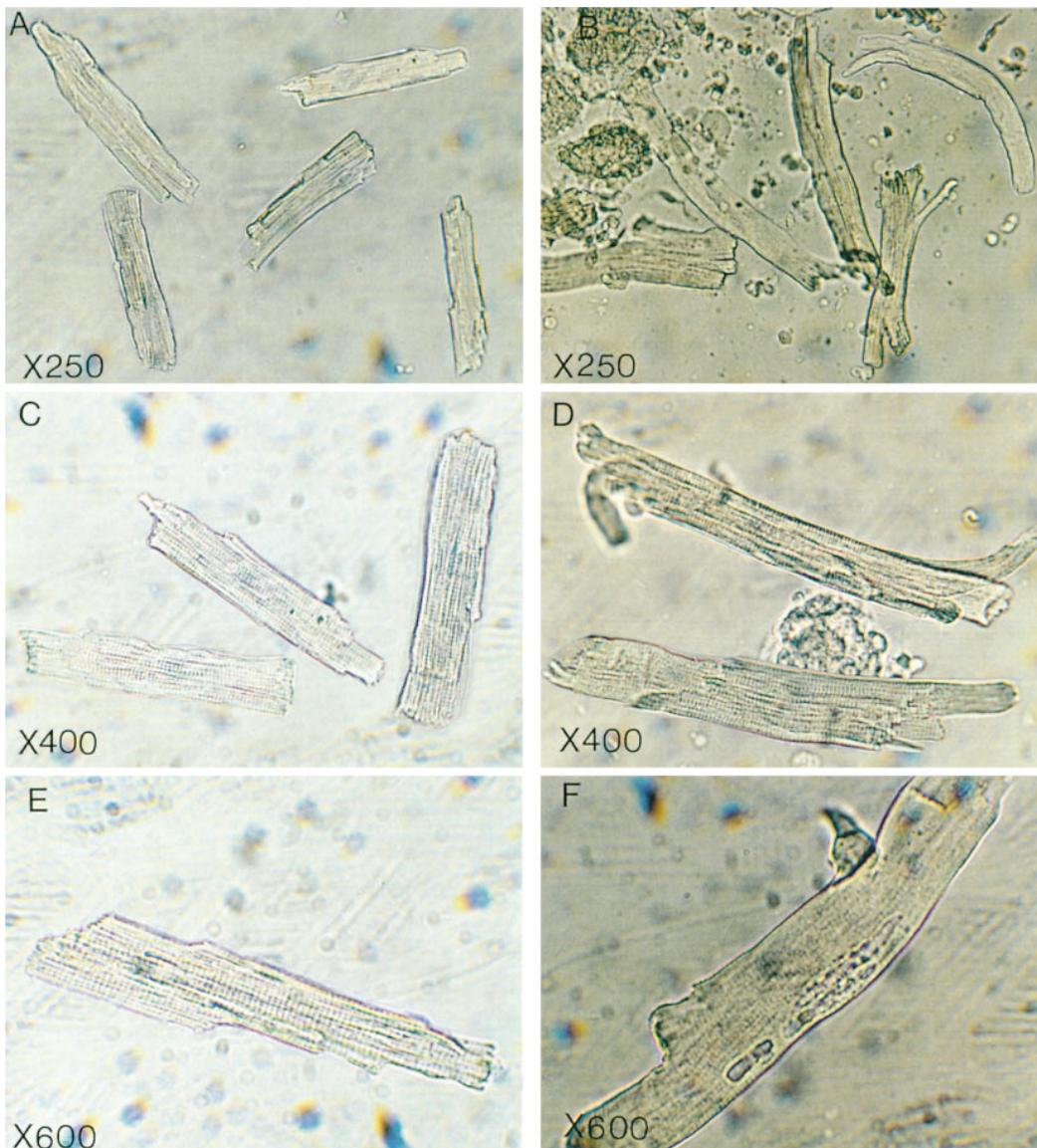
into adulthood, develop cardiac hypertrophy (1.9-fold increase in mass compared with their nontransgenic littermates; Table I) with focal areas of fibrosis, accompanied by rapid heart rate, increased respiratory rate, and fluid retention. Enzymatically isolated ventricular myocytes (19) from calsequestrin-overexpressing mice were significantly larger (~240 vs. 151 pF; Table I), and had a distinctive appearance with blurred striations (Fig. 2).

Ultrastructural analysis demonstrates that myocytes from hearts expressing excessive amounts of calsequestrin are filled with a large number of membrane-limited vesicles which pervade the entire cell outline (Fig. 3 A, arrows), displacing myofibrils and mitochondria. The vesicles contain a fine-grained electron-dense network (Fig. 3 B), presumably consisting of calsequestrin. The fine-grained appearance of calsequestrin within the vesicles is typical of that observed in the center of the terminal SR vesicles in skeletal muscle (17), but differs from the more clustered disposition of calsequestrin in the proximity of the feet-bearing domain of the SR membrane in skeletal and cardiac muscle.

Junctions between the SR and the surface membrane, or peripheral couplings (Fig. 3 C), and between the SR and T tubules (Fig. 3 D) are less frequent than in wild-type myocardium and have two distinguishing features (compare Fig. 3, C and D, with E). First, the junctions are smaller, due to the fact that the surface area of apposed membranes is smaller, and fewer feet (arrows) are present. Second, the junctional SR in the wild-type myocardium (Fig. 3 E) has the characteristic flattened shape and a dense periodically disposed content (25, 26) identified with calsequestrin. In the overexpressing myocardium, on the other hand, the junctional SR is enlarged, and the calsequestrin content is more dispersed (Fig. 3, C and D).

To assess the functional contribution of ryanodine receptors in transgenic myocytes, radioactive <sup>45</sup>Ca<sup>2+</sup> uptake by SR vesicles in whole heart homogenates was measured in the presence and absence of 300 μM ryanodine. Ca<sup>2+</sup> uptake in the absence of ryanodine reflects Ca<sup>2+</sup> transport by the longitudinal SR vesicles and the fraction of junctional SR vesicles with closed release channels. On the other hand, Ca<sup>2+</sup> uptake stimulated by ryanodine reflects the fraction of junctional SR vesicles with open Ca<sup>2+</sup> release channels, which are closed upon addition of ryanodine to the assay medium (18, 19). Fig. 4 shows that Ca<sup>2+</sup> uptake stimulated by ryanodine decreased greatly in calsequestrin-overexpressing compared with control heart homogenates. This finding is consistent with the observed downregulation of the proteins involved in the Ca<sup>2+</sup>-release cascade (ryanodine receptor, triadin, and junctin; Fig. 1 and Table I), and strongly suggests the functional impairment of Ca<sup>2+</sup>-release apparatus in transgenic hearts overexpressing calsequestrin. We observed consistently a higher level (10–25% increase) of total Ca<sup>2+</sup> uptake in homogenates from transgenic mice, which could reflect the slightly increased content of Ca<sup>2+</sup> pumps (Fig. 1) or the expanded SR volume (Fig. 3) in these animals.

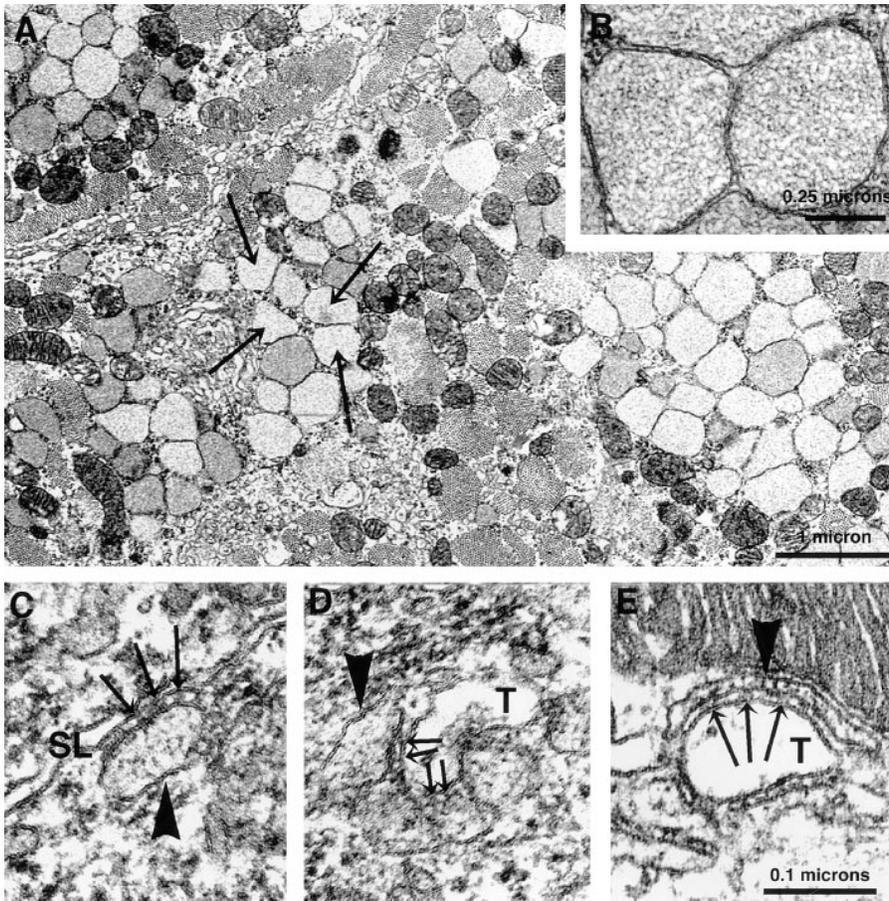
*Caffeine-induced Ca<sup>2+</sup> release is enhanced by calsequestrin overexpression.* In enzymatically isolated ventricular myocytes (21) from calsequestrin-overexpressing transgenic mice, strikingly large caffeine-induced Ca<sup>2+</sup> releases could be triggered (Fig. 5), which often saturated the Ca<sup>2+</sup> indicator dye (0.1 mM fura-2), suggesting that large Ca<sup>2+</sup> concentrations were available in the diadic junctions for release. In the vast majority of myocytes, the impressively larger caffeine-induced Ca<sup>2+</sup> release damaged irreversibly the myocytes. Nevertheless, before loss of giga-seal, large and rapidly activating  $I_{Na/Ca}$  levels were



**Figure 2.** Comparison of ventricular myocytes dissociated from control and calsequestrin-overexpressing transgenic mice. (A, C, and E) Control myocytes showing clear and regular striations. (B, D, and F) Hypertrophied and distorted calsequestrin-overexpressing myocytes at the indicated magnification.

recorded (Fig. 5 A). In calsequestrin-overexpressing myocytes dialyzed with 0.1 mM fura-2, the caffeine-induced intracellular  $\text{Ca}^{2+}$  transients ( $\text{Ca}_i$ -transients), though significantly underestimated because of saturation of the dye, were  $2.1 \pm 0.3 \mu\text{M}$  (SEM,  $n = 9$ ) activating peak  $I_{\text{Na/Ca}}$  of  $6.1 \pm 0.8 \text{ pA/pF}$  (SEM,  $n = 11$ ). In control myocytes, caffeine-induced  $\text{Ca}_i$ -transients averaged only  $0.9 \pm 0.3 \mu\text{M}$  activating peak  $I_{\text{Na/Ca}}$  of  $1.5 \pm 0.2 \text{ pA/pF}$ . Thus,  $I_{\text{Na/Ca}}$  levels in calsequestrin-overexpressing myocytes were not only much larger than in controls but also than those of transgenic mice overexpressing the cardiac  $\text{Na}^+$ - $\text{Ca}^{2+}$  exchanger ( $\sim 5 \text{ pA/pF}$ ) (20). In addition to the significantly larger (two- to fourfold) peak values of  $\Delta[\text{Ca}^{2+}]_i$  and  $I_{\text{Na/Ca}}$  in transgenic myocytes, it was apparent that both  $\text{Ca}_i$ -transients and  $I_{\text{Na/Ca}}$  remained at much higher levels above baseline values after the exposure of myocytes to caffeine, suggesting a much larger increase in total  $\text{Ca}^{2+}$  released as well as  $\text{Ca}^{2+}$  transported via the  $\text{Na}^+$ - $\text{Ca}^{2+}$  exchanger in transgenic myocytes. In fact, integration of the areas under the curves in Fig. 3 A showed that the total caffeine-induced  $\text{Ca}^{2+}$  release was

increased by  $\sim 10$ -fold in the calsequestrin-overexpressing mice, consistent with the 10-fold increase in the level of overexpression of the protein (Fig. 1). Surprisingly, large densities of  $I_{\text{Na/Ca}}$  continued to be activated in transgenic myocytes ( $n = 14$ ) even when myocytes were dialyzed with 2 mM fura-2 (Fig. 5 B). These findings suggest that in calsequestrin-overexpressing transgenic myocytes,  $[\text{Ca}^{2+}]_i$  in the microdomain surrounding the  $\text{Na}^+$ - $\text{Ca}^{2+}$  exchanger may rise to a sufficiently high level to withstand the strong  $\text{Ca}^{2+}$  buffering imposed by fura-2. The ability of calsequestrin-overexpressing myocytes to generate large  $I_{\text{Na/Ca}}$  when dialyzed with high concentrations of fura-2 was in sharp contrast not only to the control myocytes but also to those obtained from the  $\text{Na}^+$ - $\text{Ca}^{2+}$  exchanger-overexpressing myocytes (20), underscoring the possibility that  $\text{Ca}^{2+}$  concentrations reaching the microenvironment of the  $\text{Na}^+$ - $\text{Ca}^{2+}$  exchanger in calsequestrin-overexpressing myocytes were significantly larger than those estimated from the cytosolic fura-2 fluorescence signals. These are the first data that not only directly implicate calsequestrin as the store of the contractile-

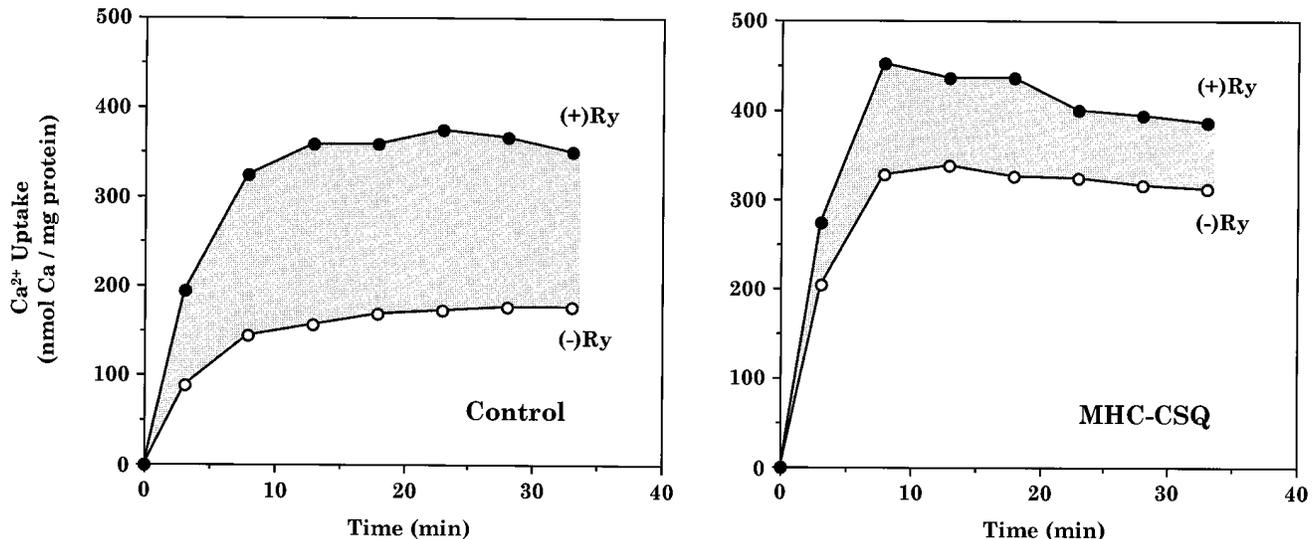


**Figure 3.** Electron microscopic analysis of SR in control and calsequestrin-overexpressing hearts. (A) Transverse section through a myocyte in the left ventricle of a calsequestrin-overexpressing mouse at 4 wk of age. At least half of the cell outline is filled by profiles of membrane-limited vesicles (arrows) with a fine content. Myofibrils and mitochondria have a less orderly arrangement than in wild-type mice (not shown). (B) Detail of two calsequestrin-containing vesicles in the experimental animal. Note that the vesicles are filled entirely by a delicate network of protein, resembling the content of the terminal cisternae of skeletal muscle, where calsequestrin is located. (C and D) Peripheral coupling (C) and dyad (D) in calsequestrin-overexpressing myocardium. The SR (arrowhead) is joined either to the surface membrane (SL) or to the T tubules (T) by feet (arrows). The SR cisternae are dilated and filled with calsequestrin. (E) Dyad in a myocyte from a wild-type mouse. The junction is more extensive, containing more feet, and the SR vesicle (arrowhead) is considerably more flat. Its calsequestrin content is more clumped than in C and D.

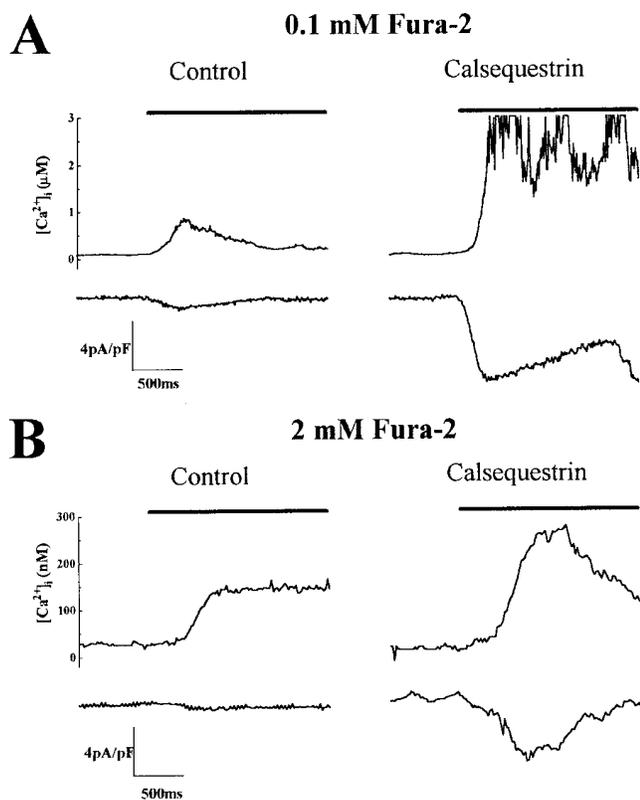
dependent pool of  $\text{Ca}^{2+}$ , but also suggest that this  $\text{Ca}^{2+}$  store can be regulated dynamically by the amount of calsequestrin that is targeted to the  $\text{Ca}^{2+}$ -release sites.

*$\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release is impaired by calsequestrin overexpression.* In sharp contrast to caffeine-triggered  $\text{Ca}^{2+}$

release,  $\text{Ca}^{2+}$  current ( $I_{\text{Ca}}$ )-induced  $\text{Ca}^{2+}$  release was significantly smaller in transgenic compared with control myocytes (Fig. 6). Depending in part on various  $\text{Ca}^{2+}$ -buffering conditions,  $\text{Ca}_i$ -transients were two to six times smaller in transgenic compared with control myocytes (Table I). The ratio of  $I_{\text{Ca}}$ -



**Figure 4.**  $\text{Ca}^{2+}$  uptake in homogenates from control and MHC-calsequestrin (CSQ) mouse ventricles. Ventricular tissue from control and transgenic hearts was homogenized. Active  $\text{Ca}^{2+}$  transport was determined in the presence or absence of 300  $\mu\text{M}$  ryanodine ( $\pm\text{Ry}$ ).



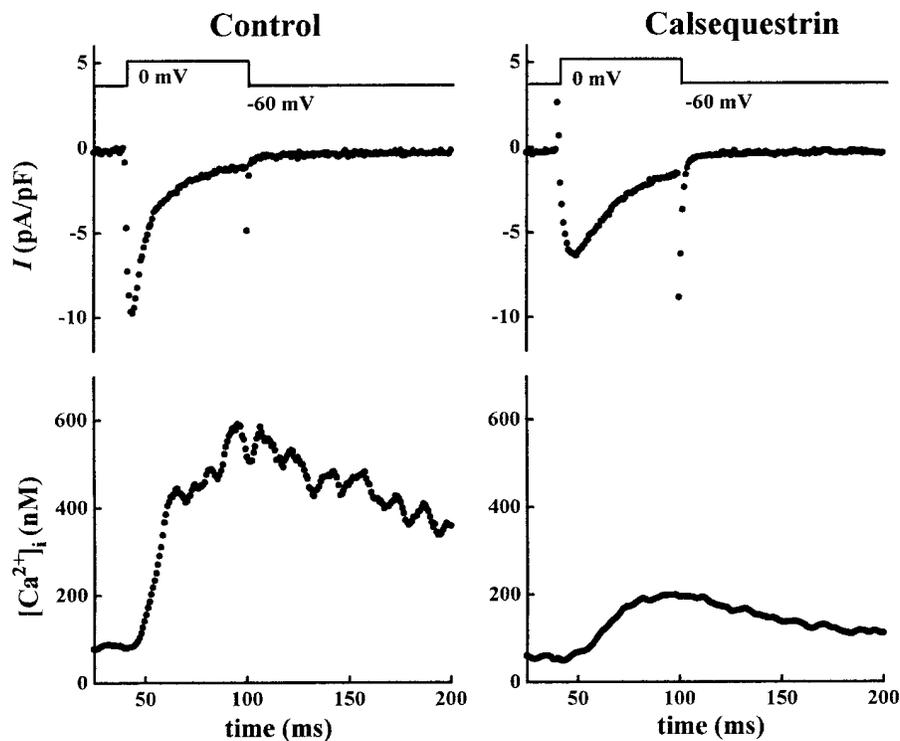
**Figure 5.** Caffeine-induced  $\text{Ca}_i$ -transients and  $I_{\text{Na}/\text{Ca}}$  in control and transgenic myocytes dialyzed with fura-2.  $I_{\text{Na}/\text{Ca}}$  was activated by  $\text{Ca}^{2+}$  release from the SR triggered by rapid application of 5 mM caffeine at a holding potential of  $-90$  mV. (A)  $\text{Ca}_i$ -transients (top) and  $I_{\text{Na}/\text{Ca}}$  (bottom) recorded from representative control and calsequestrin-overexpressing myocytes dialyzed with 0.1 mM fura-2. Bar, Timing of caffeine application. (B)  $\text{Ca}_i$ -transients (top) and  $I_{\text{Na}/\text{Ca}}$  (bottom) re-

corded from representative control and calsequestrin-overexpressing myocytes dialyzed with 2 mM fura-2. Bar, Timing of caffeine application.

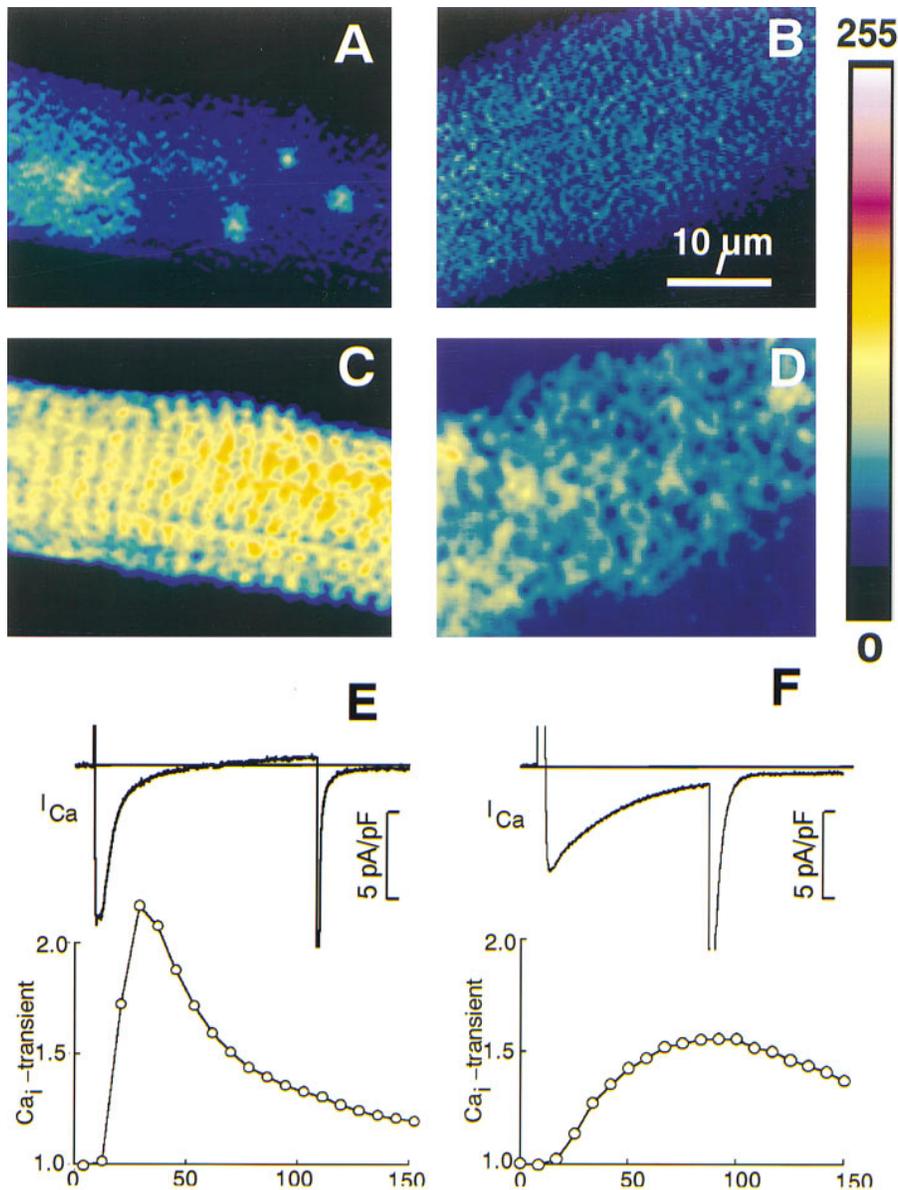
induced  $\text{Ca}^{2+}$  release to caffeine-triggered  $\text{Ca}^{2+}$  release was 0.02 in calsequestrin-overexpressing myocytes compared with 0.47 in control myocytes, suggesting an  $\sim 20$ -fold decrease in the efficiency of  $I_{\text{Ca}}$ -gated  $\text{Ca}^{2+}$  release in calsequestrin myocytes.

The inability of the  $\text{Ca}^{2+}$  channel to trigger effectively the intracellular pools was also apparent from the marked decrease in the inactivation kinetics of  $I_{\text{Ca}}$  (Fig. 6). The time constant of inactivation of  $I_{\text{Ca}}$  was two to four times greater in transgenic compared with control myocytes (irrespective of concentrations of  $\text{Ca}^{2+}$  buffers used), even though the amplitude of  $I_{\text{Ca}}$  did not change significantly (Table I). It has been suggested previously that the kinetics of inactivation of the  $\text{Ca}^{2+}$  channel can serve as a good barometer of  $\text{Ca}^{2+}$  content of the SR. Consistent with this idea, marked decreases in the kinetics of inactivation of  $I_{\text{Ca}}$  were observed in control myocytes when the SR pools were  $\text{Ca}^{2+}$ -depleted either by caffeine or thapsigargin (23, 27), a finding attributed to suppression of  $\text{Ca}^{2+}$  cross-signaling between the  $\text{Ca}^{2+}$  channel and the ryanodine receptor. In transgenic myocytes with overloaded  $\text{Ca}^{2+}$  stores (Fig. 5), the absence of  $\text{Ca}^{2+}$ -induced inactivation of the  $\text{Ca}^{2+}$  channel (Fig. 6) not only supports the impairment of the release mechanism, but also underscores the idea that the two receptors indeed function as a complex (23, 27).

The efficacy of the  $\text{Ca}^{2+}$ -release complex was further examined using rapid (240 frames/s) scanning laser confocal imaging (Fig. 7). To limit the diffusion of  $\text{Ca}^{2+}$  to  $\sim 50$  nm, myocytes were dialyzed with 1 mM fluo-3 and 5 mM EGTA, which enhanced the off-kinetics of the dye and increased the spatial and



**Figure 6.**  $I_{\text{Ca}}$ -induced  $\text{Ca}_i$ -transients in control and transgenic myocytes dialyzed with 0.1 mM fura-2.  $\text{Ca}^{2+}$  channel current (top) and  $\text{Ca}^{2+}$  release from the SR (bottom) were triggered by depolarization to 0 mV from a holding potential of  $-60$  mV.  $I_{\text{Ca}}$  (top) and  $\text{Ca}_i$ -transients (bottom) recorded from representative control and calsequestrin-overexpressing myocytes.



**Figure 7.** Ca<sup>2+</sup>-release patterns measured with confocal microscopy in control (A, C, and E) and transgenic (B, D, and F) myocytes dialyzed with fluo-3. The Ca<sup>2+</sup> distributions at the holding potential of -70 mV (A and B) and shortly after voltage clamp depolarization to 0 mV (C and D). The comb-like marker in C indicates the presence of point-like releases spaced, like sarcomeres, at 2 μm. The time course of I<sub>Ca</sub> activated from -70 to 0 mV, and the normalized cellular fluorescence is shown in E and F. The slow, nonfluorescent Ca<sup>2+</sup> buffer, EGTA, served as a secondary recipient of Ca<sup>2+</sup> and helped to stabilize the resting Ca<sup>2+</sup> concentration and prevent saturation of fluo-3 (20). The transfer of Ca<sup>2+</sup> from 1 mM fluo-3 to 1 mM EGTA is seen in E as a relatively rapid decline in the Ca<sub>i</sub>-transient which already takes place during the voltage clamp pulse. Images were recorded with a confocal microscope at 120 frames/s with a pixel size of 0.207 μm and 3 × 3 filtering before plotting on a common color scale (*inset*).

temporal resolution of confocal images. Spontaneous or I<sub>Ca</sub>-induced focal releases of Ca<sup>2+</sup> (Ca<sup>2+</sup> sparks) (28) were observed at the holding potential of -70 mV and when I<sub>Ca</sub> was activated minimally at -40 or +60 mV. In resting myocytes, spontaneous focal Ca<sup>2+</sup> releases (0.5 μm in diameter and 10 ms in duration) were seen in 16 of 18 control myocytes, but were completely absent in 19 of 20 transgenic myocytes examined (compare Fig. 7, A and B). Full activation of I<sub>Ca</sub> at 0 mV in control ventricular myocytes produced a highly regular and dynamic pattern of Ca<sup>2+</sup> stripes lasting 20 ms (C and E), yielding slowly to a more homogenous rise in cytosolic Ca<sup>2+</sup>. The Ca<sup>2+</sup> distribution at 0 mV appeared as ridges or beads spaced in register with 2-μm sarcomere spacing (Ca<sup>2+</sup> stripes). On the other hand, in transgenic myocytes, intracellular Ca<sup>2+</sup> signals developed more slowly (F) and appeared after 25 ms (three frames) as a blotchy pattern (D) similar to somewhat smeared large focal releases, but failed to produce the regular and distinct pattern of Ca<sup>2+</sup> stripes. It should be noted that the inactivation of I<sub>Ca</sub> was strongly slowed (Fig. 7 F), in agreement with

the findings of Fig. 6. In transgenic myocytes, confocal imaging data show that focal Ca<sup>2+</sup> releases are initiated infrequently and with slower kinetics and different spatial distribution patterns, resulting in slowly developing and attenuated Ca<sub>i</sub>-transients (Figs. 6 and 7) suggestive of impairment of the Ca<sup>2+</sup> release complex.

## Discussion

Although the existence of calsequestrin has been known for over 25 years (29), knowledge of its physiological roles has been limited to the postulation that it may play an active role in excitation-contraction coupling based on several *in vitro* studies (9-12). The development of transgenic mouse overexpressing cardiac calsequestrin allowed, for the first time, the probing of the role of calsequestrin at the cellular level and showing that this protein is closely and functionally linked to the Ca<sup>2+</sup>-release process. Since caffeine-induced Ca<sup>2+</sup> release

and  $\text{Na}^+$ - $\text{Ca}^{2+}$  exchanger activity are increased dramatically, and since such an increase is proportional to the degree of overexpression of calsequestrin, the results strongly implicate calsequestrin as the store of releasable  $\text{Ca}^{2+}$ .

Unexpectedly, transgenic hearts adapt to calsequestrin overexpression by altering the levels of gene expression of other proteins of the  $\text{Ca}^{2+}$ -signaling pathway, such as ryanodine receptor, triadin, and junctin. Recently, *in vitro* studies have suggested that these proteins, plus calsequestrin, associate together as a complex at the SR junction (8), and additional data have implicated calsequestrin-junctin protein interactions as important for normal operation of the  $\text{Ca}^{2+}$ -release process (30, 31). It is not yet clear whether overexpression of calsequestrin is the direct stimulus in downregulation of these proteins, or whether their level of expression is somehow linked to the functional efficacy of the  $\text{Ca}^{2+}$  channel-ryanodine receptor complex (Figs. 6 and 7). For instance, the downregulation of triadin (Fig. 1), postulated as a linking protein in the functional organization of the dihydropyridine-ryanodine receptor complex (32), is consistent with such a possibility. However, this cannot alone explain the observed failure of the  $I_{\text{Ca}}$ -induced  $\text{Ca}^{2+}$  release mechanism, because in transgenic myocytes bathed continuously in 5 mM caffeine or exposed to isoproterenol, a partial restoration of  $I_{\text{Ca}}$ -induced  $\text{Ca}^{2+}$  release could be observed (our unpublished observation). Therefore, the  $\text{Ca}^{2+}$  load of the SR may in itself serve as a determining factor in the negative feedback regulation of the  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$ -release mechanism. Such a possibility is supported by recent findings that the ryanodine receptor activity may be inactivated by high luminal  $\text{Ca}^{2+}$  concentrations (33, 34). Impairment of  $I_{\text{Ca}}$ -induced  $\text{Ca}^{2+}$  release may in part result from the distortion and/or decrease in the number of diadic junctions (Fig. 3). It should be noted that impairment of  $\text{Ca}^{2+}$  channel-gated  $\text{Ca}^{2+}$  release in the transgenic myocytes is not accompanied by compensatory activation of  $\text{Ca}^{2+}$  release by the exchanger at +10 to +40 mV, even though the exchanger functions very effectively to extrude  $\text{Ca}^{2+}$  when faced with a large increase in  $[\text{Ca}^{2+}]_i$  (Fig. 5). Therefore, the large magnitudes of caffeine-induced  $I_{\text{Na/Ca}}$  in calsequestrin-overexpressing myocytes must be secondary to the large caffeine-triggered  $\text{Ca}^{2+}$  releases, rather than compensatory overexpression of the exchanger.

Irrespective of an exact molecular mechanism responsible for impairment of  $I_{\text{Ca}}$ -induced  $\text{Ca}^{2+}$  release, the overexpression of calsequestrin appears to modulate the  $\text{Ca}^{2+}$  load of the SR and its physiological release mechanism, suggestive of a dynamic role for calsequestrin in cellular  $\text{Ca}^{2+}$  cycling. Our data further suggest that the twofold cardiac hypertrophy (Table I) is likely to result from a significant increase in the size of individual cardiac myocytes (Fig. 2). The marked increase in the number of the calsequestrin-containing vesicles (Fig. 2), though it may contribute to the increased heart weight and its structural deformity (Fig. 3), is unlikely to change the cellular capacitance, because the vesicles are confined to the intracellular space, with little or no sarcolemmal connections. However, it is possible that the observed increase in myocyte size results in part from the unique adaptive response to overexpression of calsequestrin rather than from an increase in myofibrillar mass. Whether the cytosolic calsequestrin-containing vesicles have limited access to  $\text{Ca}^{2+}$ -release pools also remains to be determined. However, it should be noted that such pools appear to be unresponsive only to the  $\text{Ca}^{2+}$  current (Figs. 6

and 7) and not to the application of caffeine (Fig. 4), even though both stimuli are known to have a shared site of action on the ryanodine receptor. This finding is therefore more consistent with a defect in dihydropyridine/ryanodine receptor signaling resulting either from altered cytoskeletal spacing that surrounds the cellular microdomains of the  $\text{Ca}^{2+}$ -release complex or from a change in the sensitivity of the  $\text{Ca}^{2+}$  regulatory site on the ryanodine receptor.

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