

Na⁺–Ca²⁺ exchange and sarcoplasmic reticular Ca²⁺ regulation in ventricular myocytes from transgenic mice overexpressing the Na⁺–Ca²⁺ exchanger

Cesare M. N. Terracciano, Ayesha I. De Souza, Kenneth D. Philipson* and Kenneth T. MacLeod

*Imperial College School of Medicine, National Heart and Lung Institute, Dovehouse Street, London SW3 6LY, UK and *Department of Physiology and Medicine, University of California Los Angeles, School of Medicine, Los Angeles, CA 90095-1760, USA*

(Received 10 March 1998; accepted after revision 28 July 1998)

1. The contribution of the sarcoplasmic reticulum (SR) and Na⁺–Ca²⁺ exchanger to intracellular Ca²⁺ regulation in mouse cardiac myocytes was investigated by measuring contraction after variable rest intervals, rapid cooling contractures (RCCs) and fast application of caffeine. The results obtained showed differences from other species in the roles played by the SR and the Na⁺–Ca²⁺ exchanger. They suggest that in mouse ventricular myocytes there is significant Ca²⁺ entry via the exchanger during rest and during the latter part of the Ca²⁺ transient.
2. In cardiac myocytes isolated from transgenic mice overexpressing the cardiac Na⁺–Ca²⁺ exchanger the time to peak and relaxation of twitches and RCCs were faster than in control littermates. The decline of Ca²⁺, assessed by indo-1 fluorescence, was faster in transgenic myocytes even in the absence of Na⁺ and Ca²⁺ in the superfusing solution. This suggests that SR Ca²⁺ uptake is faster in these myocytes. However, no difference in the expression of SERCA2a, phospholamban or calsequestrin measured with Western blotting could be found in the two groups.
3. We measured SR Ca²⁺ content by integrating the caffeine-induced transient inward current. The amount of Ca²⁺ stored in the SR of transgenic mouse myocytes was 69% greater than in non-transgenic littermates. The increased SR Ca²⁺ content may be responsible for the faster rate of SR Ca²⁺ release and uptake in cells from transgenic mice.
4. We performed experiments to assess whether the reversal potential of the Na⁺–Ca²⁺ exchanger (E_{Na-Ca}) was different in transgenic cardiac cells. We measured a Ni²⁺-sensitive current elicited by voltage ramps in non-dialysed myocytes. The current–voltage relationship showed no difference in the reversal potential of the Na⁺–Ca²⁺ exchanger in transgenic and control myocytes. This suggests that the effects on the SR Ca²⁺ content in transgenic cardiac myocytes can be ascribed to the overexpression of the exchanger and are not secondary to changes in intracellular diastolic Ca²⁺ and Na⁺.

Sarcoplasmic reticulum (SR) Ca²⁺ content is one of the major determinants of contraction in cardiac muscle (for a review see Bers, 1991). The amount of Ca²⁺ stored in the SR determines, for a certain amount of trigger Ca²⁺, the amount of Ca²⁺ released ('gain' of E–C coupling; Bassani *et al.* 1995; Janczewski *et al.* 1995) and ultimately the size of contraction. This explains several physiological findings, one being the relationship between the SR Ca²⁺ content and the rest decay demonstrated in rabbit (Bers, 1989) and guinea-pig cardiac muscle (Terracciano *et al.* 1995). The SR Ca²⁺ content also accounts for the force–frequency behaviour demonstrated in several species (Bers, 1991) and determines the rate of SR Ca²⁺ release (Terracciano *et al.* 1995), thereby regulating the

speed of contraction. The SR Ca²⁺ content primarily depends on the balance between the amount of Ca²⁺ released and the amount re-sequestered by the SR. These, in turn, are dependent on the amount of Ca²⁺ entry from the extracellular space (e.g. Bers, 1985; Bassani *et al.* 1994; Terracciano & MacLeod, 1997a) and competition with other cytoplasmic Ca²⁺ extrusion mechanisms, such as the Na⁺–Ca²⁺ exchanger.

The SR Ca²⁺ content depends on the duration of the action potential (Terracciano *et al.* 1997) suggesting that voltage-dependent mechanisms can be involved in the regulation of SR Ca²⁺ content. Two obvious voltage-dependent routes for Ca²⁺ entry into the cell are (1) via sarcolemmal Ca²⁺ channels and (2) via reverse mode Na⁺–Ca²⁺ exchange. As

the action potential is prolonged there may be more Ca^{2+} entry via sarcolemmal Ca^{2+} channels and since the cells are at more positive membrane potentials for longer periods reverse mode exchange will be favoured and help to load the SR. In other words, there may be a close dependence of SR Ca^{2+} content on the activity of the Na^+ - Ca^{2+} exchanger. Examples of this are the relationship between intracellular $[\text{Na}^+]$ and contraction (e.g. Eisner, 1990) and the mechanism of action of cardiac glycosides (Lee, 1985). In the latter case, a link between Na^+ pump blockade, a shift in the Na^+ - Ca^{2+} exchanger activity and increased SR Ca^{2+} content has been shown (Bers & Bridge, 1988). The relationship between Na^+ - Ca^{2+} exchange and cardiac relaxation is very well known (Bers, 1991; Bassani *et al.* 1994).

Finally, recent reports have shown that expression of the cardiac Na^+ - Ca^{2+} exchanger is increased (Studer *et al.* 1994; Hasenfuss *et al.* 1997) in some pathophysiological conditions, such as cardiac hypertrophy and failure, despite a slower rate of relaxation (Siri *et al.* 1991; Beuckelmann *et al.* 1992; Naqvi & MacLeod, 1994). Preliminary observations have shown that changes in intracellular Na^+ may also alter the function of the exchanger despite its overexpression (Pieske *et al.* 1997; Gray *et al.* 1997).

We have used transgenic mice overexpressing the Na^+ - Ca^{2+} exchanger to investigate the effects of the overexpression of the Na^+ - Ca^{2+} exchanger on SR Ca^{2+} release, SR Ca^{2+} uptake and the resulting SR Ca^{2+} content. The initial characterization of the effects of overexpression of the Na^+ - Ca^{2+} exchanger on contraction has recently been described (Adachi-Akahane *et al.* 1997; Yao *et al.* 1998).

METHODS

Transgenic mice overexpressing the Na^+ - Ca^{2+} exchanger

Production and characterization of transgenic (TR) mice overexpressing the Na^+ - Ca^{2+} exchanger have been described in detail elsewhere (Adachi-Akahane *et al.* 1997). In brief, a transgene construct consisting of the open reading frame of the canine cardiac Na^+ - Ca^{2+} exchanger (Nicoll *et al.* 1990) under the control of the α -myosin heavy chain (α -MHC) promoter was used. The SV40 transcriptional terminator was inserted downstream of α -MHC to provide a polyadenylation signal. The transgene was purified and microinjected into the nuclei of C57Bl/6xC3HF1 mice. Southern blot analysis of genomic DNA extracted from tail clippings was used to demonstrate the presence of the construct (TR mice). Littermates which did not show the transgene construct were used as control (NON mice).

Adachi-Akahane *et al.* (1997), using Northern blot, Western blot analysis and indirect immunofluorescent labelling, have shown that mice produced with this method overexpress the Na^+ - Ca^{2+} exchanger in the heart. They have also investigated the function of the exchanger and shown that exchanger-mediated Ca^{2+} transport is increased threefold in both inward and outward modes in cardiac myocytes isolated from the TR compared with NON mice.

Protein determination

Ventricular tissue was removed from the hearts of TR and NON mice. Samples were immediately frozen in liquid nitrogen and stored at -80°C . The frozen ventricular samples were ground in

liquid nitrogen using a pestle and mortar. Protein concentrations were assessed using a Bio-Rad protein assay followed by standard Western blot techniques. Proteins were first separated by SDS-PAGE followed by blotting onto a nitrocellulose membrane (Hybond C, Amersham). Membranes were then exposed, after a blocking step, to primary antibodies specific for the protein under investigation.

Na^+ - Ca^{2+} exchanger. To study the protein expression of the Na^+ - Ca^{2+} exchanger a primary polyclonal rabbit antiserum raised against canine sarcolemmal Na^+ - Ca^{2+} exchanger (SWant, Bellinzona, Switzerland) (1:1000) was used. The secondary antibody was an anti-rabbit Ig, peroxidase-linked, species-specific, whole antibody from donkey (Amersham) (1:2000).

SERCA2a. The primary antibody used to recognize this protein was monoclonal (mouse) anti-SERCA2 antibody (IgG2A) (Affinity Bioreagents, Inc., Golden, CO, USA) (1:5000). The secondary antibody was an anti-mouse Ig, peroxidase-linked, species-specific F(ab')₂ fragment from sheep (Amersham) (1:2000).

Phospholamban. The primary antibody used to detect this protein was a monoclonal mouse anti-phospholamban antibody (Affinity Bioreagent, Inc.) (1:10000). The secondary antibody was identical to that used to detect the anti-SERCA2a described above (1:10000).

Calsequestrin. The primary antibody was polyclonal rabbit antiserum raised against calsequestrin purified from the sarcoplasmic reticulum of dog myocardium (SWant) (1:5000). The secondary antibody was the same as that used to detect the Na^+ - Ca^{2+} exchanger (1:2000).

Secondary antibodies were detected by autoradiography using the Enhanced Chemiluminescence (ECL) system (Amersham). The films produced were then scanned and the optical densities of the bands quantified using the software package Quantity One (Pdi, New York). The values were normalized to the optical density of total protein visualized on the membrane using Amino Black (0.05% Amido Black in destain (45% H_2O , 45% methanol, 10% acetic acid)).

Cell isolation

Isolation of mice ventricular myocytes has been performed following a method previously described for the isolation of guinea-pig and rat ventricular myocytes (Terracciano & MacLeod, 1997a, b). Mice were killed by cervical dislocation and the hearts were rapidly removed and placed in ice-cold normal Tyrode (NT) solution (for the composition of this and other solutions see the Solutions section below). The ascending aorta was cannulated with a blunted 21 g needle. The heart was then retrogradely perfused for 5 min at 37°C with NT solution followed by a further 5 min in low Ca^{2+} (LC) solution. The solution was changed to an enzyme solution (ES) containing protease (4 U ml^{-1} , Sigma) for 1 min, followed by perfusion for 8 min with ES solution containing collagenase (0.8 mg ml^{-1} ; Worthington) and hyaluronidase (0.5 mg ml^{-1} , Sigma). The ventricles were then removed and cut into small pieces using a scalpel blade. The tissue was then agitated for a further 5 min in collagenase and hyaluronidase, filtered and resuspended in ES solution. This suspension was gently triturated using a glass pipette, filtered again and resuspended in ES solution. Myocytes were stored in ES solution at room temperature and used within 7–8 h of isolation.

Cell shortening measurements

Cell shortening was measured using a video-based motion detector described by Steadman *et al.* (1988). Measurements of the cell size

and length were not made, and therefore the shortening of the cell will be expressed as an absolute value and not as a percentage of cell length.

Calcium measurements

We used the Ca^{2+} -sensitive, single excitation, dual emission fluorescent dye, indo-1 AM, as described previously (Terracciano & MacLeod, 1997b). Calibration of indo-1 fluorescence was performed. The K_d was assessed *in vitro* using solutions with different [free Ca^{2+}] and a constant amount of indo-1. We previously calculated (Terracciano & MacLeod, 1997b) a K_d of 226.9 ± 2.6 nM in control conditions at pH 7.2, which compares favourably with the one reported by Grynkiewicz *et al.* (1985) (250 nM). The R_{\min} , R_{\max} and b (ratio of free to bound indo-1 fluorescence at 485 nm) were obtained *in vivo* using cells loaded with indo-1 AM. Intracellular $[\text{Ca}^{2+}]$ was varied by using the non-fluorescent Ca^{2+} ionophore 4BrA-23187 (Molecular Probes) in solutions of varying $[\text{Ca}^{2+}]$. In four cells the ratio of the final values in 'zero Ca^{2+} ' (R_{\min}) was 0.125 ± 0.01 ; the ratio in 'high Ca^{2+} ' (R_{\max}) was 0.392 ± 0.02 . The value of b was 2.584 ± 0.51 . The values for R_{\min} and R_{\max} were substantially different from the ones recorded previously in guinea-pig myocytes (0.283 and 0.878, respectively; Terracciano & MacLeod, 1997b) presumably because of different binding of the indicator to cellular proteins (Hove-Madsen & Bers, 1992).

Electrophysiology

The electrophysiological experiments were performed using an Axoclamp-2A system (Axon Instruments). To avoid dialysis of the cells and to minimize the effects of changing the intracellular environment, high resistance microelectrodes were used. In all the experiments described, borosilicate glass microelectrodes (Clark Electromedical Instruments, Reading, UK) with resistances between 20 and 30 M Ω were used.

The microelectrode filling solution contained: KCl, 2 M; EGTA; 0.1 mM; Hepes, 5 mM; pH 7.2. Unless otherwise stated the cells were stimulated in current clamp mode at 0.5 Hz with a 1.0 nA pulse of depolarizing current of 10 ms duration. Voltage-clamp experiments were performed in switch-clamp mode. The switching rate was 3–5 kHz. Gain was increased up to 0.8 nA mV^{-1} to obtain a maximally square voltage trace with no oscillations. The phase lag control was not employed in order to avoid 'false clamp'. When the cells were not impaled with microelectrodes (for example, in experiments involving rapid cooling), they were field-stimulated using a pair of platinum wires in the superfusing chamber (volume, 60 μl).

When integration of caffeine-induced transient inward currents was performed, the baseline was taken at the end of the caffeine pulse, as reported in previous papers (Terracciano *et al.* 1995, 1997).

Current clamp and voltage clamp protocols were controlled with pCLAMP 6.0.3 software (Axon Instruments).

Acquisition systems and statistical analysis

The data obtained from the video edge-detection system, the epifluorescence apparatus and the Axoclamp-2A system were simultaneously recorded on tape and on computer. The acquisition software programs used were pCLAMP 6.0.3 and Axotape 2.0 (Axon Instruments). The rate of sampling was between 0.5 and 3 kHz.

Time to peak (TTP) was measured between the point before the initial increase of the trace and the top of the signal. Time to 50% relaxation (t_{50}) was measured between the top of the signal and the

point on the declining phase corresponding to half of the total size of the twitch. To assess statistical differences between means, Student's *t* test was performed. Unless otherwise specified, the results are expressed as means \pm standard error of the mean (S.E.M.); *n* represents the number of myocytes that have undergone investigation. They were isolated from a minimum of three hearts per *n*.

Solutions

Normal Tyrode (NT) solution contained (mM): NaCl, 140; KCl, 6; MgCl_2 , 1; CaCl_2 , 1; glucose, 10; Hepes, 10; pH adjusted to 7.4 with 2 M NaOH.

Low calcium (LC) solution contained (mM): NaCl, 120; KCl, 5.4; MgSO_4 , 5; CaCl_2 , 0.045; sodium pyruvate, 5; glucose, 20; taurine, 20; Hepes, 10; nitrotriacetic acid (NTA), 5; bubbled with 100% O_2 ; pH 6.8.

Enzyme solution (ES) contained (mM): NaCl, 120; KCl, 5.4; MgSO_4 , 5; CaCl_2 , 0.2; sodium pyruvate, 5; glucose, 20; taurine, 20; Hepes, 10; bubbled with 100% O_2 ; pH 7.4.

Na^+ -free- Ca^{2+} -free solution contained (mM): LiCl, 140; KOH, 6; MgCl_2 , 1; glucose, 10; Hepes, 10; EGTA, 0.1; pH adjusted to 7.4 with 1 M LiOH.

Chemicals were generally purchased from BDH. Nifedipine, 4-aminopyridine, glibenclamide and niflumic acid were from Sigma and thapsigargin from Calbiochem.

The temperature of the superfusing solution was approximately 22 °C in control conditions. The rate of superfusion was 2–3 ml min^{-1} except during rapid cooling or fast application of caffeine when it was 12–15 ml min^{-1} . Miniature solenoid valves (The Lee Company, Essex, UK) were used to produce fast changes in superfusate.

RESULTS

The $\text{Na}^+ - \text{Ca}^{2+}$ exchanger in the E–C coupling of control mouse ventricular myocytes

There have been many studies on the relative contribution of various cellular mechanisms to cytoplasmic Ca^{2+} regulation in different species (see Bers, 1991), but very little is known about such Ca^{2+} regulation in mouse ventricular myocytes. Before investigating the effects of the overexpression of the $\text{Na}^+ - \text{Ca}^{2+}$ exchanger on SR Ca^{2+} regulation, we performed experiments to assess how the exchanger contributes to Ca^{2+} regulation of control mouse ventricular myocytes. We carried out three groups of experiments: measurements of Ca^{2+} transients after variable rest intervals, rapid cooling contractures and caffeine application. The results differ substantially from previous studies in cardiac tissue of other species so we shall describe them first to provide necessary background information before showing the results obtained from transgenic myocytes.

Post-rest contraction

Measurements of contraction after variable periods of rest in mouse ventricular myocytes have been described before (He *et al.* 1997). We have repeated these experiments and demonstrated a similar behaviour in the myocytes isolated in our laboratory. Figure 1A shows that prolonging the rest interval increases the amplitude of the post-rest contraction

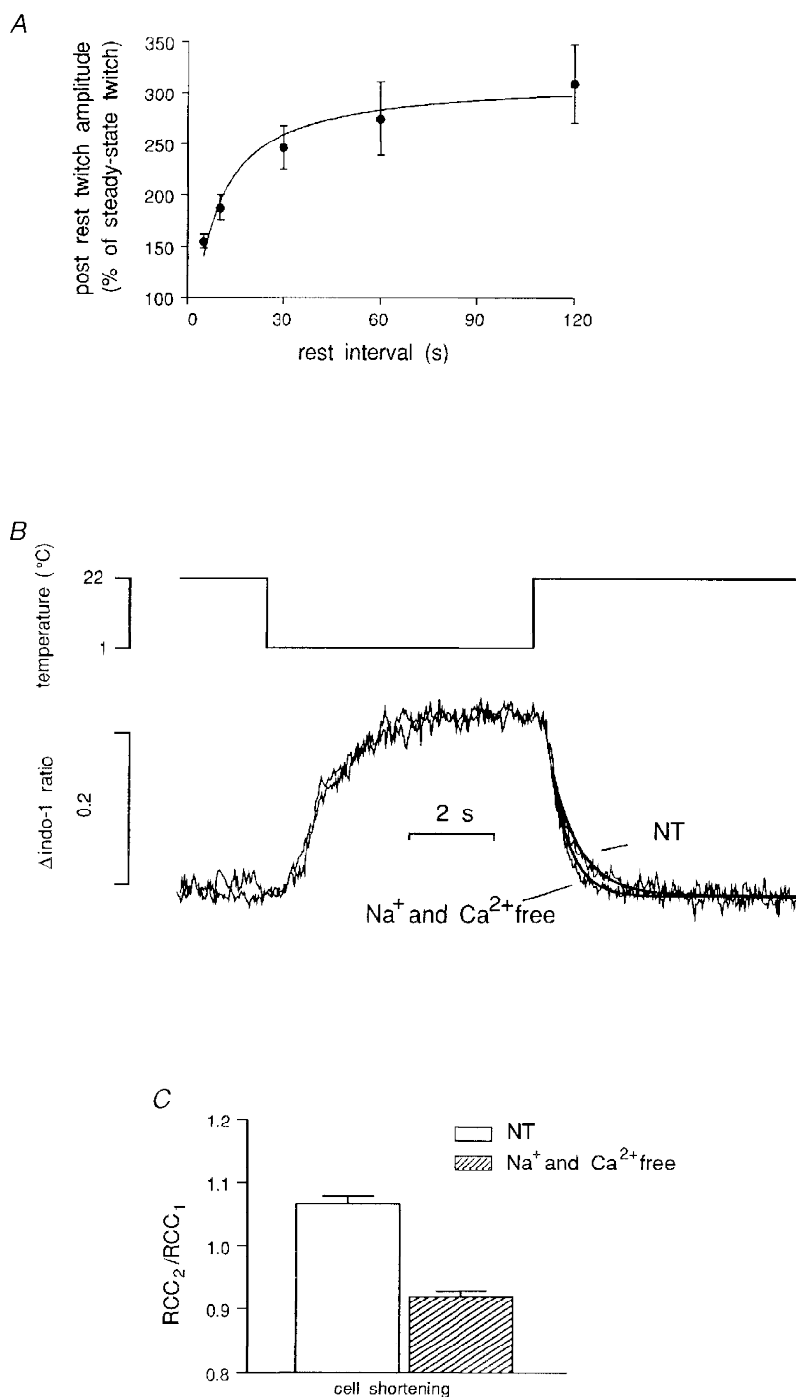


Figure 1. The effects of rest and rapid cooling on cell shortening and indo-1 fluorescence in control mouse ventricular myocytes

A, rest produces potentiation of the twitch. Cells were stimulated at 0.5 Hz until they reached steady-state contraction and then rested for variable intervals. The data are means \pm s.e.m. of 12 myocytes (7 for 60 s interval and 5 for 120 s). Several cells showed spontaneous contractions during prolonged rest. *B*, indo-1 fluorescence during rapid cooling followed by rewarming. In this case no calculation of $[Ca^{2+}]_i$ was performed since the higher level of fluorescence was close to saturation and the cooling alters the K_d of the dye. The cell was initially stimulated at 0.5 Hz. Stimulation was stopped for 5 s before applying rapid cooling. Traces from a RCC performed in NT solution and in Na⁺- and Ca²⁺-free solution have been superimposed. In the latter case Na⁺- and Ca²⁺-free solution was used from cessation of stimulation throughout rapid cooling and rewarming until relaxation was completed. Lines were fitted on the declining phase of the indo-1 trace with the equation $A \exp[-(t - K)/\tau] + C$ where A is the amplitude of the signal,

(rest-potential). This behaviour suggests that in mouse myocytes the balance between Ca^{2+} entry and Ca^{2+} extrusion during rest is in favour of the former, thus increasing the Ca^{2+} content of the SR (in contrast with guinea-pig and rabbit which show rest decay).

Rapid cooling contractures (RCCs)

Rapid cooling of cardiac muscle to 1 °C in less than 1 s produces a contracture (Kurihara & Sakai, 1985) due to the release of Ca^{2+} from the SR (Bridge, 1986). The phenomenon has been ascribed to the effects of temperature on the gating of ryanodine receptors (Sitsapesan *et al.* 1991). This technique allows qualitative assessment the SR Ca^{2+} content and study of the relative contributions of various mechanisms to Ca^{2+} regulation. A Na^+ - and Ca^{2+} -free solution can be used during rewarming after rapid cooling to eliminate the contribution of the $\text{Na}^+-\text{Ca}^{2+}$ exchanger to relaxation. This solution is used only during cooling and rewarming in order to leave the SR loading unaffected. Assuming a minimal role for other extrusion mechanisms, such as the sarcolemmal Ca^{2+} -ATPase and mitochondria, rewarming in the absence of external Ca^{2+} and Na^+ can be used to assess the function of SR Ca^{2+} uptake during relaxation. Figure 1B shows traces of indo-1 fluorescence changes during rapid cooling and rewarming in NT or Na^+ - and Ca^{2+} -free solution. Both traces reach values close to saturation and therefore in this case no calculation of $[\text{Ca}^{2+}]_i$ can be made. Surprisingly, during rewarming after the rapid cooling performed in Na^+ - and Ca^{2+} -free solution, the time constant τ of the curves fitted to the lower part of the indo-1 fluorescence decline was smaller than the one recorded during normal Tyrode (NT) superfusion (τ of indo-1 ratio decline in NT: 725 ± 92 ms, $n = 7$; τ in Na^+ - and Ca^{2+} -free solution: 409 ± 56 ms, $n = 7$; $P = 0.005$). This is in contrast with results obtained in other species, such as guinea-pig (Terracciano & MacLeod, 1997a) and rabbit (Bassani *et al.* 1994) where relaxation was prolonged in these conditions. A possible explanation for this phenomenon is that in mouse ventricular myocytes the $\text{Na}^+-\text{Ca}^{2+}$ exchanger does not contribute to Ca^{2+} extrusion during the lower part of the Ca^{2+} transient. During this period the $\text{Na}^+-\text{Ca}^{2+}$ exchanger may reverse to take Ca^{2+} into the cytoplasm. Its functional inhibition would therefore lead to the diastolic level being reached more quickly.

To investigate this phenomenon further we performed paired RCCs. This technique has been described in detail

before (Hryshko *et al.* 1989; Terracciano & MacLeod, 1997b). Ca^{2+} taken up by the SR during rewarming after the first RCC is assessed by measuring the amplitude of a second RCC elicited after a fixed interval and comparing this with the size of the first RCC. If the $\text{Na}^+-\text{Ca}^{2+}$ exchanger activity is inhibited by superfusing the cell with a Na^+ - and Ca^{2+} -free solution throughout the whole period the amplitude of the second RCC represents the amount of Ca^{2+} taken up by the SR plus the amount of Ca^{2+} handled by the exchanger. Figure 1C shows that maximal contractures during the second cooling in NT solution were larger than the first RCCs suggesting a net increase in the Ca^{2+} taken up into the SR during rewarming after the first RCC.

The origin of this Ca^{2+} seems to be extracellular since removing Ca^{2+} from the superfusing solution significantly reduces $\text{RCC}_2/\text{RCC}_1$. During RCCs only a small change in resting membrane potential occurs due to the effects of temperature on the Nernst equation (Bridge, 1986; Naqvi *et al.* 1994). This small depolarization is not likely to be able to increase the open probability of the sarcolemmal Ca^{2+} channels and therefore this route for Ca^{2+} entry should be ruled out. Assuming no non-specific Ca^{2+} leak, the only mechanism able to produce this Ca^{2+} entry is the $\text{Na}^+-\text{Ca}^{2+}$ exchanger working in outward current mode (i.e. Ca^{2+} influx- Na^+ efflux producing net outward current).

Fast application of caffeine

Fast application of caffeine is another method used to study $\text{Na}^+-\text{Ca}^{2+}$ exchanger function. Rapid application of 10 mM caffeine produces a maximal release of Ca^{2+} from the SR. If the superfusion with caffeine is maintained, relaxation is brought about by non-SR mechanisms, mainly $\text{Na}^+-\text{Ca}^{2+}$ exchange (Varro *et al.* 1993; Bassani *et al.* 1994). In addition, measuring the transient inward current produced by application of caffeine has been widely used to assess the amount of Ca^{2+} stored in the SR (Varro *et al.* 1993; Terracciano & MacLeod, 1997a).

We performed these experiments in control mouse myocytes to address two questions: (1) how much Ca^{2+} is stored in the SR of control mouse ventricular myocytes? (2) How much do the minor Ca^{2+} extrusion mechanisms contribute to relaxation in the absence of a functionally intact SR? Figure 2A (left side) shows that application of caffeine produces a contraction (top) and a transient inward current. Note that indo-1 fluorescence was not used in these experiments in order to avoid adding extra Ca^{2+} buffering to the cytoplasm.

C is the offset, $t - K$ is the time interval between the start and the end of the fit and τ is the time constant. The rate of decline of the indo-1 ratio during rewarming in Na^+ - and Ca^{2+} -free solution was faster than in NT solution. C , paired RCC experiments. A second RCC (RCC_2) was performed 10 s after a first RCC (RCC_1) as described above and no stimulation was given during this time. The ratio $\text{RCC}_2/\text{RCC}_1$ represents the amount of Ca^{2+} handled by the SR during rewarming compared with the previous RCC. These data obtained by measuring cell shortening changes show that in control mouse myocytes, more Ca^{2+} than that released during the first cooling period is reaccumulated into the SR during the rewarming phase. Removing Na^+ and Ca^{2+} from the superfusing solution abolishes this effect.

We tested the Na^+ and Ca^{2+} dependency of the caffeine-induced current by repeating the same experiment in a Na^+ - and Ca^{2+} -free solution. As shown in Fig. 2A (right) in this condition, although a maximal contraction was still present, the transient inward current was almost abolished suggesting that the current we measured (and shown in the left side of the figure) is dependent on the Na^+ - Ca^{2+} exchanger.

An important factor that influences the calculation of the SR Ca^{2+} content assessed using this method is the amount of Ca^{2+} extruded by mechanisms other than the Na^+ - Ca^{2+} exchanger. To assess the importance of such mechanisms, we repeated similar experiments but we also measured $[\text{Ca}^{2+}]_i$ changes using indo-1 fluorescence. As observed in the trace

in Fig. 2A, in the absence of a functionally intact SR and Na^+ - Ca^{2+} exchanger, relaxation after caffeine application was extremely slow. Data for τ of the decline of $[\text{Ca}^{2+}]_i$ are shown in Fig. 2B. τ of Ca^{2+} decline in caffeine was 2582 ± 266 ms ($n = 10$), a value about 2.7 and 1.6 times greater than the one previously measured in rabbit and rat ventricular myocytes, respectively, with a similar technique (Bassani *et al.* 1994). The τ for decline of $[\text{Ca}^{2+}]_i$ in caffeine and Na^+ - and Ca^{2+} -free solution was $31\,645 \pm 6525$ ms ($n = 10$), approximately 2.6 times slower than in rat and rabbit myocytes measured with the same technique by Bassani *et al.* (1994). This suggests that, if other minor mechanisms are involved in relaxation, their role is minimal.

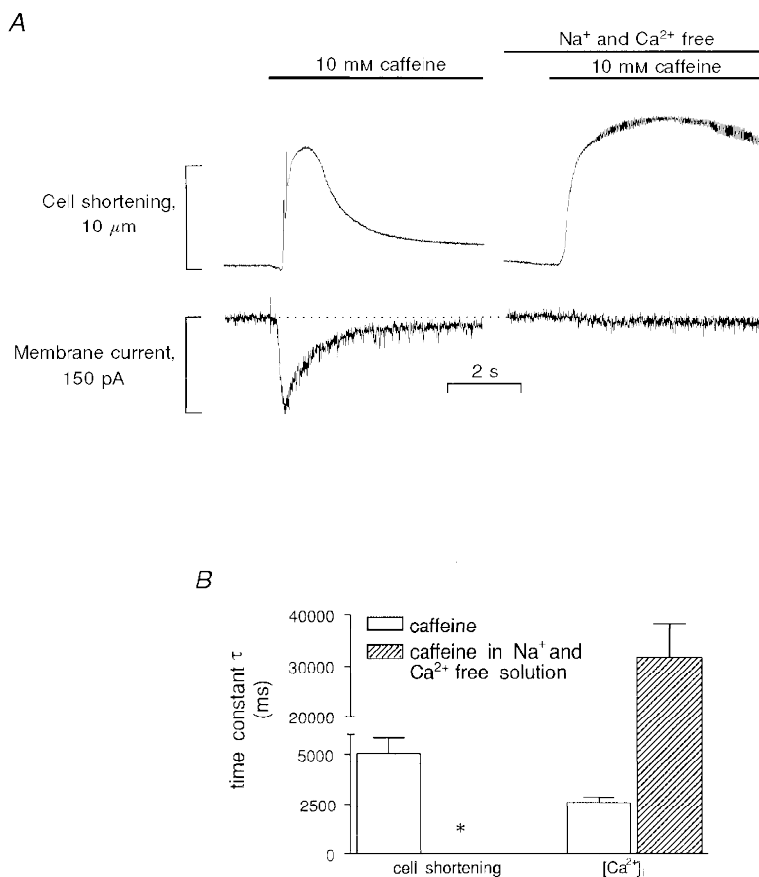


Figure 2. The effect of fast application of 10 mM caffeine on cell shortening, membrane current and intracellular $[\text{Ca}^{2+}]_i$ in control mouse myocytes

Cells were initially stimulated at 0.5 Hz (not shown) and after 2 s rest, caffeine was rapidly applied and its application maintained for several seconds. A, left side, a typical caffeine-induced transient inward current. The integral of this current allows calculation of the amount of Ca^{2+} released from the SR. A, right side, a similar caffeine application in the same cell but in the absence of Na^+ and Ca^{2+} in the superfusate. Although a large release of Ca^{2+} is still present (as demonstrated by the contracture induced by caffeine) the current was almost abolished suggesting that it can be ascribed to the Na^+ - Ca^{2+} exchanger. Note that in the absence of Na^+ and Ca^{2+} in the superfusate, relaxation is extremely slow and cannot be accurately measured (*). In many cases it was not possible to determine a time constant of relaxation since the cell seems to stay contracted. Data are shown in B along with data from cells loaded with indo-1. This was done to allow a more direct measurement of Ca^{2+} extrusion. No indo-1 was used when these experiments were employed to calculate SR Ca^{2+} content.

Table 1. Ca^{2+} transients and twitches in TR and NON ventricular myocytes

	TR	NON	<i>P</i> (<i>t</i> test)
Diastolic $[\text{Ca}^{2+}]$ (nM)	116 ± 8 (18)	127 ± 11 (13)	0.45
Ca^{2+} transient amplitude (nM)	168 ± 17 (20)	241 ± 57 (9)	0.127
Ca^{2+} transient TTP (ms)	441 ± 34 (20)	663 ± 46 (9)	0.001
Ca^{2+} transient t_{50} (ms)	329 ± 18 (20)	455 ± 22 (9)	0.0004
Twitch amplitude (μm)	4.6 ± 0.8 (20)	3.2 ± 0.3 (8)	0.317
Twitch TTP (ms)	472 ± 35 (20)	658 ± 85 (9)	0.022
Twitch t_{50} (ms)	404 ± 35 (20)	639 ± 86 (9)	0.005

Values of *n* given in parentheses.

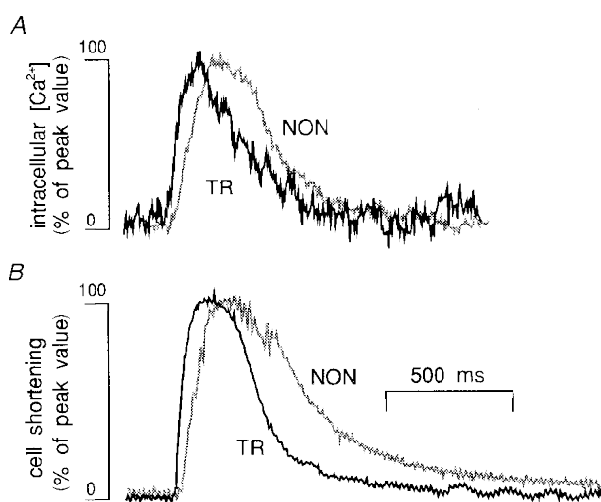
In conclusion we have demonstrated that in mouse ventricular myocytes the $\text{Na}^+-\text{Ca}^{2+}$ exchanger plays a minor role in relaxation compared with other species; it seems that it is involved in Ca^{2+} entry during rest and perhaps during the latter part of relaxation.

RCCs in transgenic mice overexpressing the $\text{Na}^+-\text{Ca}^{2+}$ exchanger

Contraction and relaxation in transgenic mice overexpressing the $\text{Na}^+-\text{Ca}^{2+}$ exchanger has been previously described (Adachi-Akahane *et al.* 1997). We also measured cell shortening and Ca^{2+} transients elicited by field stimulation at 0.5 Hz in TR and NON myocytes (Fig. 3). Ca^{2+} transients and twitches were of similar size in the two groups but the rates of contraction and relaxation were significantly faster in TR than in NON myocytes (Table 1).

These results confirm findings previously reported by Adachi-Akahane *et al.* (1997) who ascribed the faster relaxation to a larger Ca^{2+} extrusion brought about by the

$\text{Na}^+-\text{Ca}^{2+}$ exchanger. To investigate whether changes in SR Ca^{2+} uptake can also be responsible for such a faster relaxation, we performed RCCs. Figure 4A shows traces of Ca^{2+} decline during rewarming after RCCs performed using NT solution in TR and NON myocytes. Ca^{2+} decline in TR myocytes was faster than in NON myocytes. This is consistent with the findings observed in twitches. Nevertheless, as for twitches, RCCs in NT solution do not enable us to distinguish between the different relaxation mechanisms. To investigate specifically SR Ca^{2+} uptake in Ca^{2+} removal, we performed RCCs in Na^+ - and Ca^{2+} -free solution. A typical experiment is shown in Fig. 4B. Data for Ca^{2+} changes during RCCs are reported in Table 2 (amplitude is not shown because during RCCs the indo-1 fluorescence reaches values close to saturation). The rate of decline of Ca^{2+} in TR myocytes was faster than the one measured in NON myocytes. This suggests that the faster decline of Ca^{2+} during rewarming in TR myocytes can also be due to faster SR Ca^{2+} uptake.

**Figure 3.** Ca^{2+} transients in TR and NON ventricular myocytes

Typical Ca^{2+} transients (A) and twitches (B) recorded in TR (black traces) and NON (grey traces) myocytes elicited by field stimulation at 0.5 Hz. Traces were scaled and superimposed to compare the time courses of the signals. Both changes in cell length and intracellular $[\text{Ca}^{2+}]$ were faster in TR than in NON myocytes.

Table 2. Ca^{2+} decline during rewarming after RCCs in TR and NON myocytes

	TR	NON	<i>P</i> (<i>t</i> test), TR vs. NON
NT solution, RCCs			
t_{50} (ms)	859 ± 56 (20)	1051 ± 74 (15)	0.043
τ (ms)	768 ± 61 (21)	1016 ± 101 (13)	0.033
Na^+ - and Ca^{2+} -free solution			
t_{50} (ms)	579 ± 46 (20)	742 ± 42 (13)	0.021
τ (ms)	519 ± 35 (20)	673 ± 60 (12)	0.025
NT solution, Ca^{2+} increase			
τ (ms)	256 ± 20 (19)	657 ± 92 (12)	< 0.0001

Values of *n* given in parentheses.

We analysed the rate of increase of Ca^{2+} during the onset of rapid cooling (Fig. 4C). τ of the mono-exponential curve, fitted on the increasing phase of the Ca^{2+} trace during cooling, was smaller in TR than in NON myocytes; data are

shown in Table 2. This suggests that upon cooling in TR myocytes, Ca^{2+} is released faster from the SR and may account for the faster TTP of twitches shown in the previous section.

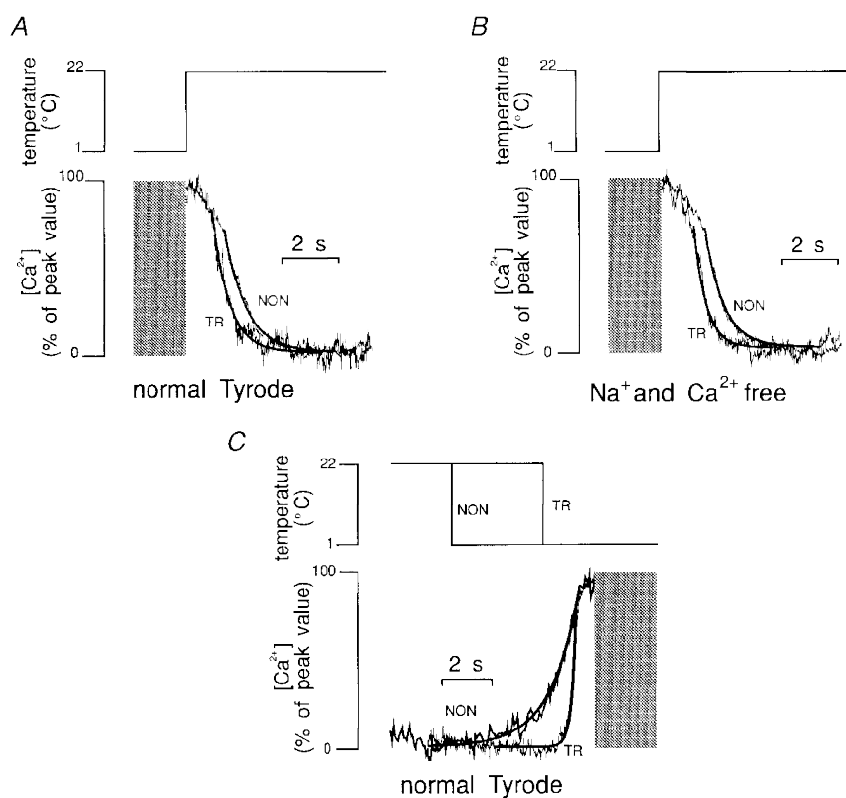


Figure 4. The effects of rapid cooling and rewarming on TR and NON myocytes

Traces have been scaled then superimposed and finally lines were fitted using the equation described in the legend of Fig. 1. Since indo-1 fluorescence reached values close to saturation during maximal Ca^{2+} release, the top parts of the traces are not shown (grey rectangles). *A*, Ca^{2+} decline recorded from a TR myocyte in NT was faster than the one recorded in a NON myocyte. *B*, similar findings were observed when rewarming was performed in Na^+ - and Ca^{2+} -free solution although both traces were significantly faster than the ones recorded in NT solution. *C*, increase in $[\text{Ca}^{2+}]_i$ upon cooling. Traces were scaled then superimposed such that the peak values coincide. The rate of increase in TR myocytes was faster than the one recorded in NON myocytes.

In conclusion, in TR myocytes, even when Na⁺-Ca²⁺ exchange function is prevented by removing the Na⁺ and Ca²⁺ gradient, Ca²⁺ removal from the cytoplasm is still faster, presumably due to a faster SR Ca²⁺ uptake. To explain this phenomenon several hypotheses can be put forward. We first tested the possibility of an alteration of SR Ca²⁺ uptake due to concomitant changes in the expression of SR proteins.

Ca²⁺ regulatory protein determination in transgenic mouse cardiac tissue overexpressing the Na⁺-Ca²⁺ exchanger

We used Western blotting to assess the level of Ca²⁺ regulatory proteins associated with the SR, namely SERCA2a, phospholamban and calsequestrin. We did this to assess whether changes in protein expression in TR myocytes occur as an adaptive response to an overexpression of Na⁺-Ca²⁺ exchanger or as a consequence of the transgene construct. We observed a strong immunoreactivity, as expected, in TR tissue when we used anti-Na⁺-Ca²⁺ exchanger antibodies. The results were comparable to those described by Adachi-Akahane *et al.* (1997) and confirmed overexpression of the exchanger. Nevertheless, as is evident from Fig. 5, we did not detect significant differences in any of the experiments testing the immunoreactivity of other antibodies in TR or NON tissue. This suggests that in TR cardiac tissue the changes to SR

Ca²⁺ uptake described in the previous section cannot be ascribed to changes in the expression of SR proteins.

Measurement of the SR Ca²⁺ content in non-dialysed transgenic mouse myocytes overexpressing the Na⁺-Ca²⁺ exchanger

One of the possible functional reasons for a faster relaxation after Ca²⁺ release (such as the one recorded during rewarming after a cooling period) is that more Ca²⁺ has been released in TR than in NON myocytes. As reported by Bers & Berlin (1995), there is a relationship between peak Ca²⁺ and the rate of Ca²⁺ removal from the cytoplasm. One may therefore expect a larger SR Ca²⁺ content in TR myocytes. We analysed the SR Ca²⁺ content by measuring the charge carried by the transient inward current induced by application of caffeine. We performed these experiments in non-dialysed myocytes voltage clamped at -75 mV. Figure 6A shows traces of caffeine-induced current recorded in TR and NON myocytes. The currents were of similar magnitude and showed similar kinetics compared with those described by Adachi-Akahane *et al.* (1997). Data are shown in Table 3. In TR myocytes the amplitude of the current was larger and the rate of decline was faster than in NON myocytes. We also observed a faster rate of increase and, most importantly, a significantly larger integral of the current in TR than in NON myocytes (Fig. 6B). The SR

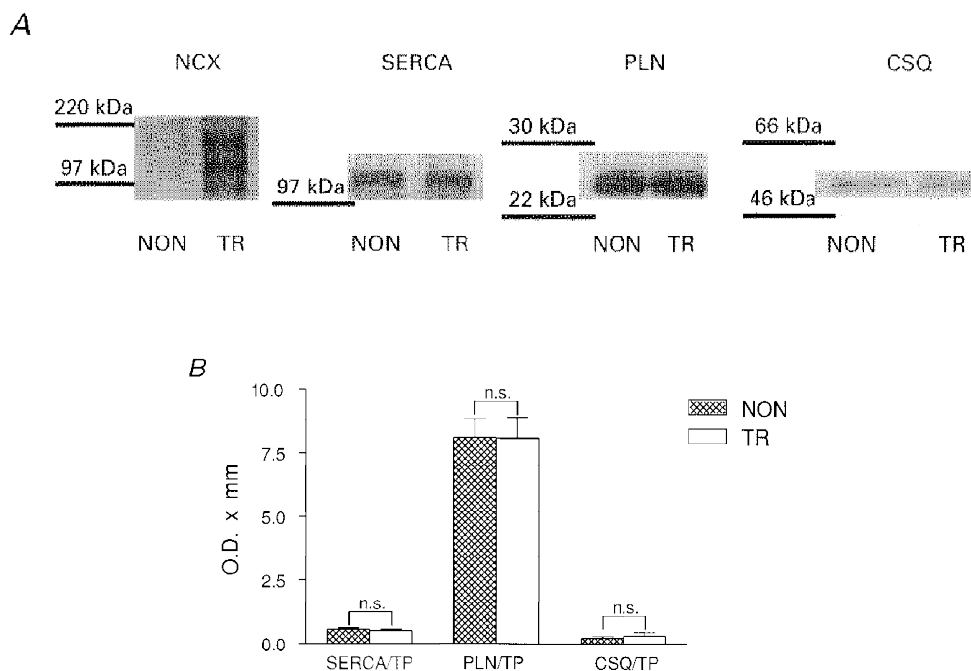


Figure 5. Measurements of the expression of Ca²⁺ regulatory proteins in TR and NON cardiac tissue using Western blotting

A, examples of Western blots for Na⁺-Ca²⁺ exchanger (NCX), SR Ca²⁺-ATPase (SERCA), phospholamban (PLN) and calsequestrin (CSQ). The results for NCX were similar to that shown by Adachi-Akahane *et al.* (1997) where bands at 160, 120 and 70 kDa were shown in TR cardiac tissue. The values for optical density of the single proteins were normalized to total protein (TP) and data are shown in *B*. Data are means \pm S.E.M., $n = 6$.

Table 3. Caffeine-induced transient inward current in TR and NON myocytes

	TR	NON	<i>P</i> (<i>t</i> test)
Cell capacitance (pF)	252 ± 15 (28)	252 ± 18 (18)	0.995
Cell volume (pl)	29.9 ± 2 (28)	29.9 ± 2 (18)	0.995
Charge (pC)	-276 ± 21 (28)	-164 ± 18 (18)	0.0006
Charge/capacitance (pC pF ⁻¹)	-1.080 ± 0.03 (28)	-0.642 ± 0.03 (18)	< 0.0001
Ca ²⁺ (μmol l ⁻¹ a.v.)†	118.1 ± 4.2 (28)	70.2 ± 4.2 (18)	< 0.0001
Current amplitude (pA)	-893 ± 85 (28)	-299 ± 42 (17)	< 0.0001
Current density (pA pF ⁻¹)	-3.47 ± 0.25 (28)	-1.16 ± 0.14 (18)	< 0.0001
Current increase (nA s ⁻¹)	-10.41 ± 1.76 (25)	-2.73 ± 0.42 (17)	0.001
Time constant (τ) of current decay (ms)	242 ± 29 (24)	525 ± 27 (16)	< 0.0001

† a.v., accessible cell volume. Values of *n* given in parentheses.

Ca²⁺ content of TR myocytes was therefore significantly larger than the one recorded in NON myocytes.

Assessment of the ability of the Na⁺-Ca²⁺ exchange to load the SR in non-dialysed transgenic mouse myocytes overexpressing the Na⁺-Ca²⁺ exchanger

From the results described in Fig. 6, it is possible to identify a relationship between the overexpression of the Na⁺-Ca²⁺ exchanger and the SR Ca²⁺ content. This represents an

important finding demonstrating that the exchanger can control SR Ca²⁺. To investigate this relationship further, we performed the experiments described in Fig. 7. They were based on the hypothesis that, if the overexpression of the Na⁺-Ca²⁺ exchanger is responsible for a larger SR Ca²⁺ content, reloading the SR after complete Ca²⁺ depletion should take place more rapidly in TR myocytes. Important assumptions in this series of experiments are that

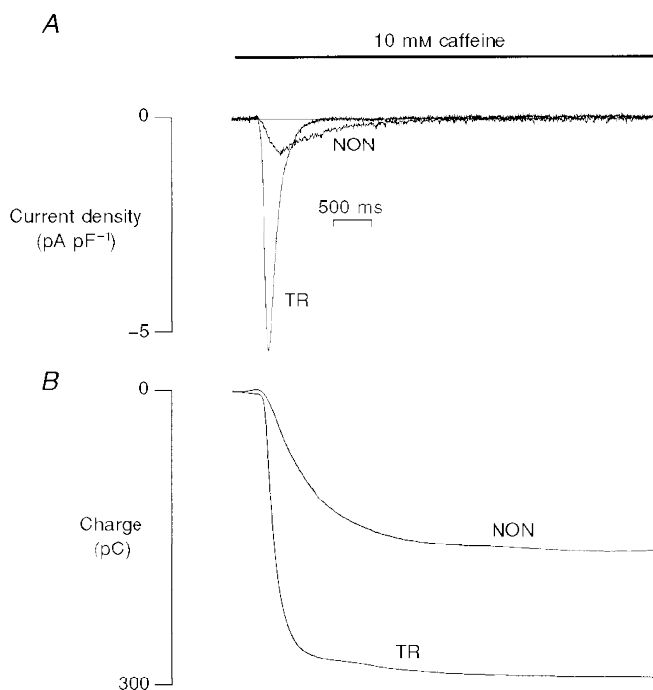


Figure 6. The effects of fast application of caffeine on membrane current in TR and NON myocytes

Cells were stimulated at 0.5 Hz until steady-state contraction was reached and then stimulation was stopped; 2 s after cessation of stimulation the cells were voltage-clamped at -75 mV. Caffeine superfusion was then started and maintained for 6 s. *A*, superimposed current density traces recorded from TR and NON myocytes. *B*, integrals of the currents. In TR myocytes the amount of charge crossing the cell membrane during caffeine application was larger and increased more rapidly than in NON myocytes.

application of caffeine depletes the SR of Ca²⁺ in both TR and NON myocytes and that, in the presence of 20 μ M nifedipine, Ca²⁺ entry is brought about mostly by the Na⁺-Ca²⁺ exchanger.

The reloading protocol consisted of voltage-clamp steps of 400 ms duration from -75 to +20 mV applied at 0.5 Hz. We chose this protocol in order to keep the voltage command constant, thus avoiding changes in action potential duration (Silverman *et al.* 1995) that could be responsible for changes of the SR Ca²⁺ load (Terracciano *et al.* 1997). We observed that in NON myocytes (Fig. 7A), after 100 steps, the SR Ca²⁺ content was still approximately half of the one measured at steady state. In contrast, in TR myocytes (Fig. 7B), the steady-state SR Ca²⁺ content was larger (as previously demonstrated) and half of this value was reached after twenty-five steps. The SR Ca²⁺ content had returned to the steady-state value after 100 steps. Data

are summarized in Fig. 8 where SR Ca²⁺ content values are expressed as absolute values and as a percentage of the steady-state content. These results suggest that during blockade of Ca²⁺ channels in TR myocytes, the Na⁺-Ca²⁺ exchanger is able to load the SR more rapidly. It therefore may be responsible for the increased SR Ca²⁺ content demonstrated in these myocytes. In NON myocytes, reloading produced by the exchanger is much slower. In these cells presumably Ca²⁺ entry via the sarcolemmal Ca²⁺ channels plays a larger role in beat-to-beat SR Ca²⁺ regulation.

Voltage-current relationship of Na⁺-Ca²⁺ exchange and determination of subsarcolemmal Na⁺ in non-dialysed transgenic mouse myocytes overexpressing the Na⁺-Ca²⁺ exchanger

From the results reported above, it is clear that the Na⁺-Ca²⁺ exchanger in TR myocytes produces functional

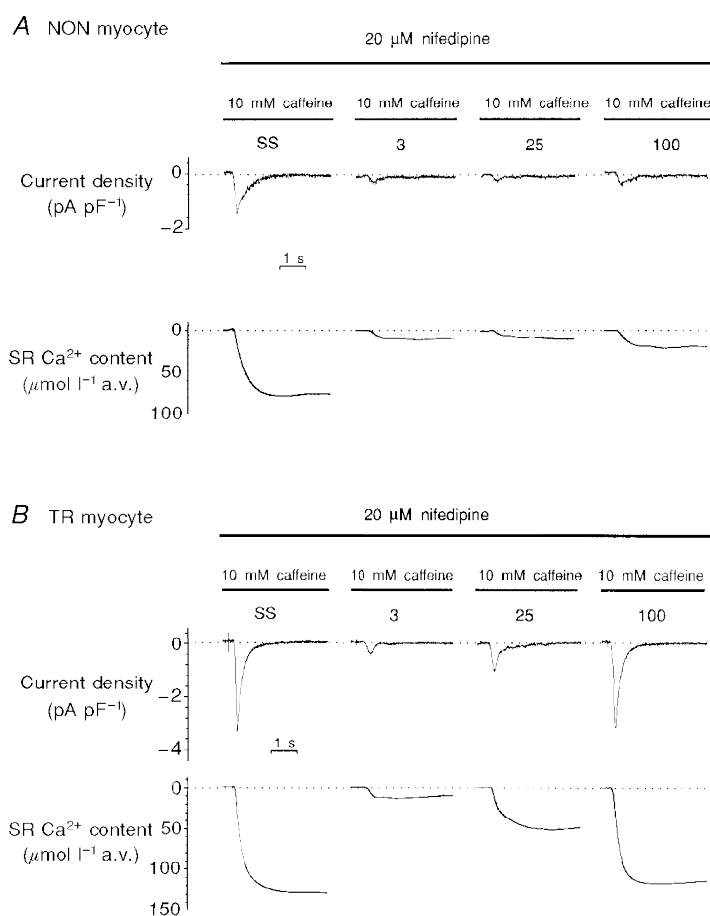


Figure 7. Reloading of the SR with increasing numbers of voltage-clamp steps in TR (A) and NON myocytes (B)

Steady-state (SS) SR Ca²⁺ content was calculated as described in Fig. 6 and Methods. Caffeine was then withdrawn and an increasing number of voltage-clamp steps (from -75 to +20 mV) of 400 ms duration at 0.5 Hz were applied. The ability of these steps to reload the SR was tested by another caffeine application. The number of steps used is shown above the caffeine traces. Caffeine was then withdrawn again and a different number of steps applied. Experiments were performed in the presence of 20 μ M nifedipine in order to abolish the contribution of L-type Ca²⁺ channels to Ca²⁺ entry.

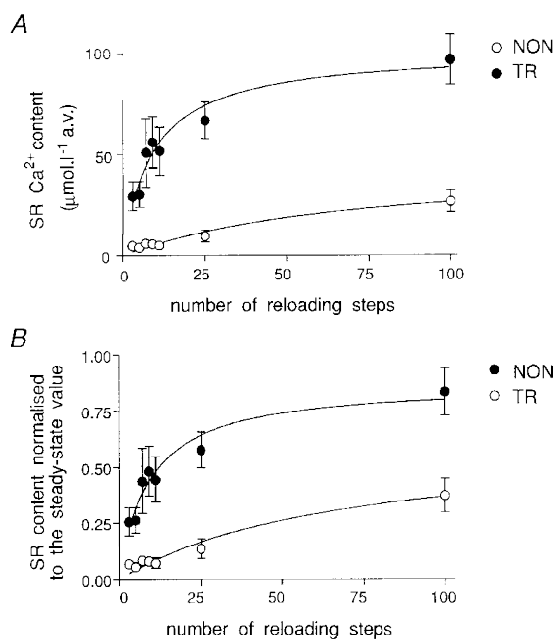


Figure 8. Reloading in TR myocytes is faster and larger than in NON myocytes

Data from the reloading experiments described in Fig. 7 ($n \geq 5$). *A*, absolute value of SR Ca²⁺ content after each set of loading steps. In *B*, these data have been normalized to the value of SR Ca²⁺ content calculated under steady-state conditions before initiating superfusion with nifedipine.

changes in SR Ca²⁺ regulation. Nevertheless, whether these effects are due to the overexpression of the Na⁺-Ca²⁺ exchanger or to changes in the equilibrium potentials (E_{Ca} and E_{Na}) governing its function is still not clear.

To investigate whether the reversal potential of the exchanger (E_{Na-Ca}) is modified in TR myocytes we performed experiments based on measuring the current-voltage relationship of the Na⁺-Ca²⁺ exchanger and determining E_{Na-Ca} in a manner similar to that described by

Kimura *et al.* (1986) and Kimura *et al.* (1987). However, our method is different in that we used high resistance microelectrodes to avoid dialysis of the cell. Under these circumstances we estimated the [Na⁺] that the exchanger 'sees' (i.e. subsarcolemmal [Na⁺]) and therefore determined whether this differs in TR myocytes. In order to block most of the conductances and isolate the Na⁺-Ca²⁺ exchanger current we superfused the cell with NT solution containing 3 mM 4-aminopyridine, 20 μM nifedipine, 2 mM BaCl₂,

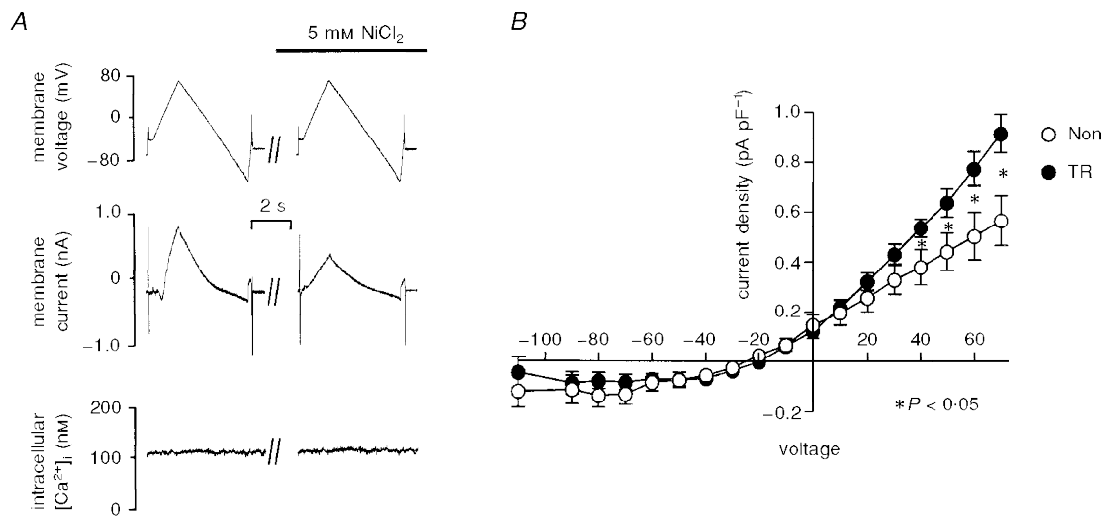


Figure 9. Current-voltage relationships of the Na⁺-Ca²⁺ exchanger current in TR and NON myocytes

A, the protocol employed to measure the current. Intracellular [Ca²⁺] was recorded simultaneously. The voltage ramp was performed in control conditions and then in the presence of 5 mM NiCl₂ (for composition of the solutions see Results). The Na⁺-Ca²⁺ exchange current was calculated by subtracting the current recorded in Ni²⁺ from the one obtained in control conditions. *B*, current-voltage relationship calculated for 12 TR and 8 NON myocytes. Current was significantly larger in TR myocytes at voltages between +40 and +70 mV. The currents reversed at approximately -25 mV in both groups.

Table 4. $E_{\text{Na-Ca}}$ exchange, intracellular $[\text{Na}^+]$ and $[\text{Ca}^{2+}]$ in TR and NON myocytes

	TR	NON	P (t test)
$E_{\text{Na-Ca}}$	-26.8 ± 3.3 (12)	-24 ± 2.7 (8)	0.55
Intracellular $[\text{Ca}^{2+}]$ (nM)	159 ± 28 (12)	135 ± 23 (8)	0.57
Intracellular $[\text{Na}^+]$ (mM)	9.6 ± 0.9 (12)	9.6 ± 0.7 (8)	0.97

Values of n given in parentheses.

2 mM CsCl, 20 μM glibenclamide and 10 μM niflumic acid. To avoid changes in intracellular Ca^{2+} during the voltage protocol, the SR was emptied by superfusion with 500 nM thapsigargin and intracellular Ca^{2+} was buffered with BAPTA AM (1 μM , Molecular Probes). We superfused cells loaded with indo-1 AM with this solution for at least 5 min before recording. The voltage protocols were similar to ones used previously (Kimura *et al.* 1986, 1987; Hobai *et al.* 1997). From a holding potential of -75 mV, we clamped membrane voltage at -40 mV for 200 ms and then performed a voltage ramp to $+80$ mV and back to -120 mV at a velocity 50 mV s^{-1} . We then rapidly switched the superfusing solution to a similar solution but containing 5 mM NiCl_2 and, after 5 s rest, repeated the ramp protocol. We recorded intracellular Ca^{2+} simultaneously. A typical example of this type of experiment is shown in the inset in Fig. 9. A Ni^{2+} -sensitive, $\text{Na}^+-\text{Ca}^{2+}$ exchange current resulted from subtraction of the current recorded during the descending phase of the ramp protocol in Ni^{2+} from the one obtained during the same phase in control. Indo-1 fluorescence was used to calculate the intracellular $[\text{Ca}^{2+}]$. Average values of $\text{Na}^+-\text{Ca}^{2+}$ exchange current in eight NON myocytes and twelve TR myocytes are shown in Fig. 9. In TR myocytes a statistically significant increase in current density was present between $+40$ and $+70$ mV compared with NON myocytes. We did not detect differences at other voltages. We did not detect changes in $E_{\text{Na-Ca}}$ and we did not observe difference in intracellular $[\text{Ca}^{2+}]$ in the two groups. Since $E_{\text{Na-Ca}} = 3E_{\text{Na}} - 2E_{\text{Ca}}$, we could calculate intracellular $[\text{Na}^+]$. The results suggest that intracellular Na^+ is the same in TR and NON myocytes at rest (Table 4). Our results suggest that no changes in cytoplasmic $[\text{Ca}^{2+}]$ and $[\text{Na}^+]$ are present in cells from the two groups and this is consistent with the results reported by Yao *et al.* (1998).

DISCUSSION

SR Ca^{2+} content and $\text{Na}^+-\text{Ca}^{2+}$ exchange in control mouse ventricular myocytes

It is now well established that the relative contributions of different mechanisms to Ca^{2+} regulation during the cardiac cycle can vary according to species (Bers, 1985, 1991). For example the relative roles played by the SR Ca^{2+} -ATPase and the $\text{Na}^+-\text{Ca}^{2+}$ exchanger are different in rabbit and guinea-pig cardiac muscle from those in rat cardiac muscle

(e.g. Bers, 1991; Terracciano & MacLeod, 1997a). In order to obtain more information on the relative contribution of these mechanisms to Ca^{2+} regulation in mouse myocardium, experiments were initially performed on control mouse myocytes before undertaking experiments on cells from transgenic animals.

The mouse cardiac myocytes show similarities with rat myocardial cells. Post-rest potentiation, for example, followed a similar time course to the one observed in rat myocardium (Bers, 1991) and a negative force-frequency relationship has already been described in mouse myocytes (Wolska & Solaro, 1996). A peculiar finding was that the time constant of rewarming after rapid cooling was faster in the combined absence of Na^+ and Ca^{2+} , suggesting that Ca^{2+} entry occurs at some point during the decline of intracellular Ca^{2+} . Although it is difficult to predict the effect of reversed $\text{Na}^+-\text{Ca}^{2+}$ exchange function on the *total time constant* of relaxation, it is possible to predict that the time constant for relaxation *after the exchanger has reversed* will be increased. This increase may be reflected in an alteration to the total time constant that we measure.

The hypothesis that Ca^{2+} entry occurs during the decline of intracellular Ca^{2+} was examined using paired RCCs. During rewarming and rest, more Ca^{2+} than that expelled from the SR during the first cooling is taken back up into the SR. This observation, together with the demonstration that the extra SR loading is inhibited by Na^+ - and Ca^{2+} -free superfusate, suggests that the $\text{Na}^+-\text{Ca}^{2+}$ exchanger is responsible for Ca^{2+} entry during the decline in Ca^{2+} . We suppose that, at the beginning of relaxation, $\text{Na}^+-\text{Ca}^{2+}$ exchange is in inward mode and extrudes Ca^{2+} from the cell but, as the intracellular $[\text{Ca}^{2+}]$ declines, the reversal potential of the $\text{Na}^+-\text{Ca}^{2+}$ exchange is reached and it ceases to remove Ca^{2+} from the cell. Instead, it reverses and takes Ca^{2+} into the cell, reducing the speed of relaxation and allowing a larger amount of Ca^{2+} to be taken up by the SR. SR uptake represents the favoured route for Ca^{2+} since at this level of cytoplasmic Ca^{2+} the contribution of the other mechanisms to relaxation is minimal.

We calculated that during steady-state contraction in control mouse ventricular myocytes the SR Ca^{2+} content was 59.7 ± 10 $\mu\text{mol l}^{-1}$ accessible cell volume (a.v.) (non-mitochondrial; Terracciano *et al.* 1995) ($n=6$). It should be noted that the absolute value of SR Ca^{2+} content calculated with

this method is dependent on the capacitance: volume ratio used. Since a value for mouse myocytes is not available, here and in all the other experiments described in this paper we have used the value calculated for rat ventricular myocytes of 8.4 pF pl^{-1} by Satoh *et al.* (1996). We decided to use the value measured for rat myocytes for two reasons: (1) the average cell volume of mouse cells calculated using this correction factor was similar to the one calculated in other species (approximately 30 pl ; see Table 3) and (2) rat myocytes have similar post-rest and force–frequency behaviour to mouse myocytes and therefore, at least as far as E–C coupling is concerned, they seem to be more similar to mouse cells than other species. However, it is possible that there are large differences in the capacitance: volume ratio between rat and mouse myocytes, and, indeed, between myocytes from TR and NON, and if this occurs it will lead to miscalculation of the SR Ca^{2+} content. This should be kept in mind when comparing results from different species and from cells from TR and NON.

SR Ca^{2+} uptake in myocytes from transgenic (TR) mice

We found that in TR mouse hearts there was more rapid Ca^{2+} removal from the cytoplasm (even in the absence of Na^+ and Ca^{2+} in the superfusing solution) than in NON hearts. Since other Ca^{2+} extrusion mechanisms play a very minimal role in relaxation in mouse myocytes, the results suggest that SR Ca^{2+} uptake is increased in TR compared with NON myocytes. We initially wondered if, in TR myocytes, there had been complementary changes to the levels of expression of other Ca^{2+} handling proteins. However, there was no evidence for changes in the expression of the major SR Ca^{2+} proteins such as SERCA2a, phospholamban or calsequestrin, which suggested that the kinetics of SR Ca^{2+} uptake had been affected in the presence of similar amounts of protein.

A larger release of Ca^{2+} from the SR (either upon rapid cooling or during electrical stimulation) could account for this phenomenon. This is because it has been shown by Bers & Berlin (1995) that increasing peak $[\text{Ca}^{2+}]_i$ produces a faster decline or recovery to diastolic values in cardiac myocytes. These authors have demonstrated that in similar conditions (absence of Na^+ and Ca^{2+} in the superfusate), Ca^{2+} decline ($\Delta\text{Ca}^{2+}/\Delta t$) can be predicted assuming that Ca^{2+} transport rate and Ca^{2+} binding depend on $[\text{free } \text{Ca}^{2+}]$. Since we have shown, by integrating the caffeine-induced currents, that the SR Ca^{2+} content is larger in TR than in NON myocytes, we expect that the peak $[\text{Ca}^{2+}]_i$ reached upon cooling is higher in the former cells (although we cannot detect it with the fluorescence experiments, presumably due to dye saturation). The faster Ca^{2+} decline observed in TR myocytes is therefore expected even in the absence of other functional or morphological changes in SR Ca^{2+} uptake.

SR Ca^{2+} content in TR myocytes

Adachi-Akahane *et al.* (1997) have already assessed SR Ca^{2+} content using changes in the peak indo-1 fluorescence

during application of caffeine. This was the first study using the transgenic mouse overexpressing the exchanger and it was anticipated that SR Ca^{2+} content would be smaller than in non-transgenic mouse heart cells because more exchanger would be available to compete with the SR Ca^{2+} -ATPase for Ca^{2+} . However, they concluded that the SR Ca^{2+} content was not significantly smaller in TR myocytes than in NON myocytes but their study did not have sufficient power to determine if the SR Ca^{2+} content was greater. Further, on complete release of SR Ca^{2+} , fura-2 fluorescence, while not perhaps reaching R_{max} , may be on the non-linear part of the fluorescence– $[\text{Ca}^{2+}]_i$ relationship and so may not give a reliable indication of the SR Ca^{2+} content.

To obtain reliable and accurate values of the SR Ca^{2+} content we integrated the caffeine-induced transient inward current. We calculated a larger SR Ca^{2+} content in TR myocytes than in NON myocytes. In order to discuss these results two important assumptions need to be made.

These are: (1) The contribution of minor cellular mechanisms (i.e. mechanisms other than the SR Ca^{2+} -ATPase and Na^+ – Ca^{2+} exchange) involved in Ca^{2+} regulation does not affect the calculation of SR Ca^{2+} content. We have shown that other Ca^{2+} extrusion mechanisms play only a very minor role in relaxation in mouse myocytes. As shown in Fig. 2, where it could be measured, the τ for decline of $[\text{Ca}^{2+}]_i$ in caffeine and Na^+ - and Ca^{2+} -free solution was approximately 2.5 times slower than in rat and rabbit myocytes measured with the same technique by Bassani *et al.* (1994). In many cases the cells did not relax when superfused with caffeine and a Na^+ - and Ca^{2+} -free solution. For this reason we did not correct for Ca^{2+} extrusion produced by these mechanisms and the values for SR Ca^{2+} content are derived purely by the integration of the caffeine-induced transient inward current.

(2) The capacitance: volume ratio used (8.4 pF pl^{-1}) is adequate for mouse myocytes and is not different in myocytes from TR and NON mice. The absolute values derived from the pure integration of the caffeine-induced transient inward current may be in error because we do not know with precision the capacitance: volume ratio of mouse cardiac ventricular myocytes.

It is probable that the increased SR content in TR myocytes occurs because of the overexpression of the exchanger. The reloading experiments (in the presence of nifedipine to inhibit Ca^{2+} influx through the L-type Ca^{2+} channel) shown in Fig. 7 suggest that in TR myocytes the Na^+ – Ca^{2+} exchanger alone is capable of reloading the SR and is thus able to regulate SR Ca^{2+} content.

In conclusion we speculate that, bearing in mind that in mouse cardiac myocytes the Na^+ – Ca^{2+} exchanger brings Ca^{2+} into the cells at low cytoplasmic $[\text{Ca}^{2+}]_i$, this Ca^{2+} entry is larger in cells with overexpression of the exchanger, thus producing a larger SR Ca^{2+} content.

SR Ca^{2+} release in TR myocytes

The increased SR Ca^{2+} content in TR myocytes can explain the previous observation that the intracellular $[\text{Ca}^{2+}]$ during electrical stimulation and rapid cooling increases more rapidly in TR than in NON myocytes. A larger SR Ca^{2+} content is able to increase the rate of SR Ca^{2+} release (Terracciano *et al.* 1995), perhaps because of the effects of increasing luminal Ca^{2+} on the properties of the SR Ca^{2+} release channels (e.g. Sitsapesan & Williams, 1997). The overexpression of the $\text{Na}^+-\text{Ca}^{2+}$ exchanger therefore indirectly increases the rate of Ca^{2+} release from the SR affecting the speed of contraction. Whether Ca^{2+} entry via the $\text{Na}^+-\text{Ca}^{2+}$ exchanger is also important in producing a faster TTP during electrically stimulated twitches in TR myocytes cannot be determined by the experiments described in this paper.

Evidence for enhanced $\text{Na}^+-\text{Ca}^{2+}$ exchanger function in TR myocytes

Adachi-Akahane *et al.* (1997) showed clear evidence of increased $\text{Na}^+-\text{Ca}^{2+}$ exchanger protein with both Western blotting and immunofluorescence. We have obtained similar results. Although a larger amount of protein is present in these animals, whether this produces a greater proportion of Ca^{2+} removal via the exchanger is more questionable. Adachi-Akahane *et al.* (1997) showed that Ca^{2+} removal from the cytoplasm after application of caffeine was larger and faster in TR than in NON myocytes suggesting an increased efflux of Ca^{2+} via the exchanger. Although our experiments yielded similar results, our interpretation of these results is slightly different.

$\text{Na}^+-\text{Ca}^{2+}$ exchanger function can only be assessed in this way when releases of Ca^{2+} are of similar magnitude. Different amounts of release produce changes in the kinetics of the exchanger resulting in different current amplitudes (Kimura *et al.* 1987). Since our results show that caffeine application to TR myocytes results in a larger SR release, then the size of the Ca^{2+} transient and associated current will be larger. Therefore the larger current observed in TR myocytes may be not only related to the overexpression of the $\text{Na}^+-\text{Ca}^{2+}$ exchanger, but also partly explained by the increase in the amount of Ca^{2+} released from the SR.

Direct evidence of increased function of the $\text{Na}^+-\text{Ca}^{2+}$ exchanger in TR myocytes comes from the measurements of Ni^{2+} -sensitive $\text{Na}^+-\text{Ca}^{2+}$ exchanger current elicited by changes in voltage elicited in a similar manner to that previously described (Kimura *et al.* 1986, 1987). With this method we were able to show that the outward current produced by the $\text{Na}^+-\text{Ca}^{2+}$ exchanger for voltages between +40 and +70 mV was significantly larger in TR than in NON myocytes. This was in the absence of changes in the reversal potential of the $\text{Na}^+-\text{Ca}^{2+}$ exchanger as discussed below and represents direct evidence of increased function of the exchanger in TR myocytes.

We did not detect differences in the current at more negative potentials where the $I-V$ relationship was almost

flat. This may have been caused by our use of non-dialysing sharp microelectrodes and by using different solutions from ones used by other investigators. However, the currents we have recorded are remarkably similar to the ones obtained using more traditional dialysing patch-clamp techniques. They also showed an almost flat configuration at negative potentials (Kimura *et al.* 1987; Hobai *et al.* 1997). Kimura *et al.* (1987) suggested that the carrier probably saturates at these potentials. Our results do not support this view since we would expect that increasing the amount of carrier (overexpression of $\text{Na}^+-\text{Ca}^{2+}$ exchanger) would produce saturation at higher current amplitude. Recently, Convery & Hancox (1998) have shown that this flattening of the current response does not seem to be dependent on saturation but on the direction of ramp used, being present only during descending voltage ramps (from positive to negative voltages). It is possible that this effect could be due to a reduction of subsarcolemmal $[\text{Ca}^{2+}]$ as negative values of potential are reached during the ramp. A reduced subsarcolemmal $[\text{Ca}^{2+}]$ will result in less inward current produced by the $\text{Na}^+-\text{Ca}^{2+}$ exchanger. If Ca^{2+} extrusion is performed more effectively in TR myocytes it sets subsarcolemmal $[\text{Ca}^{2+}]$ to a lower level, thereby further reducing the amount of current. This can explain why we do not see any difference in the two groups. All these considerations suggest that the measurement of the $\text{Na}^+-\text{Ca}^{2+}$ exchanger current at negative potentials during a descending voltage ramp can be misleading. The absolute value of $E_{\text{Na}-\text{Ca}}$ may also be misleading since intra- and extracellular $[\text{Ca}^{2+}]$ are artificial because of the buffers and blockers used in the superfusing solution. Nevertheless comparison between the $E_{\text{Na}-\text{Ca}}$ values in identical conditions is possible and it is interesting to note that $E_{\text{Na}-\text{Ca}}$ is unchanged in the two groups of myocytes, suggesting that the Na^+ regulatory mechanisms are able to maintain $[\text{Na}^+]_i$ to the same level in myocytes from TR and NON mice. We do not think that the protocol affects the more positive side of the $I-V$ curve, (Convery & Hancox (1998) showed no difference in that portion of the curve using different voltage clamp protocols) and so outward currents can provide better measurements of $\text{Na}^+-\text{Ca}^{2+}$ exchanger activity.

In myocytes from TR and NON mice, no differences can be detected in diastolic intracellular $[\text{Ca}^{2+}]$ and $[\text{Na}^+]$ (see also Adachi-Akahane *et al.* 1997; Yao *et al.* 1998). Our reversal potential experiments confirm these findings. The reasons for similar Na^+ and Ca^{2+} values are unknown. One can speculate that the same $[\text{Na}^+]$ in the two cell types arises from the ability of the Na^+ pump to control Na^+ at a set level. Similarly, the SR Ca^{2+} pump is able to maintain closely matching levels of diastolic $[\text{Ca}^{2+}]$ in the two cell types. We did not measure the activity of the SR Ca^{2+} pump directly in these myocytes at rest and therefore we assume that the affinity of the pump for Ca^{2+} is probably the same in the two cell types. If the affinities are the same, one would anticipate that an increased SR uptake in TR myocytes requires a higher diastolic $[\text{Ca}^{2+}]$. We conclude

that the difference between the diastolic $[Ca^{2+}]$ in the two cell types is smaller than can be measured with our system.

During stimulation we know that cytoplasmic $[Ca^{2+}]$ increases more quickly in myocytes from TR, and this means that Ca^{2+} is available more quickly as a substrate for the pump during the twitch. This, together with a larger release of Ca^{2+} from the SR during the twitch, may be responsible for the faster relaxation and ultimately for the increased SR Ca^{2+} content in myocytes from TR.

In conclusion, we have shown that an increased expression of the Na^+-Ca^{2+} exchanger strongly affects SR Ca^{2+} regulation, particularly by increasing SR Ca^{2+} content. This results in a faster SR Ca^{2+} release and SR Ca^{2+} uptake. From these findings it can be speculated that changes in the expression of the Na^+-Ca^{2+} exchanger protein in pathophysiological conditions can result in modifications of SR Ca^{2+} regulation in cardiac muscle.

- 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.
- BASSANI, J. W. M., BASSANI, R. A. & BERS, D. M. (1994). Relaxation in rabbit and rat cardiac cells: species-dependent differences in cellular mechanisms. *Journal of Physiology* **476**, 279–293.
- BASSANI, J. W. M., YUAN, W. & BERS, D. M. (1995). Fractional SR Ca release is regulated by trigger Ca and SR Ca content in cardiac myocytes. *American Journal of Physiology* **268**, C1313–1319.
- BERS, D. M. (1985). Ca influx and sarcoplasmic reticulum Ca release in cardiac muscle activation during post rest recovery. *American Journal of Physiology* **248**, H366–381.
- BERS, D. M. (1989). SR Ca loading in cardiac muscle preparations based on rapid-cooling contractures. *American Journal of Physiology* **256**, C109–120.
- BERS, D. M. (1991). *Excitation-Contraction Coupling and Cardiac Contractile Force*. Kluwer Academic, Dordrecht.
- BERS, D. M. & BERLIN, J. R. (1995). Kinetics of $[Ca]_i$ decline in cardiac myocytes depend on peak $[Ca]_i$. *American Journal of Physiology* **268**, C271–277.
- BERS, D. M. & BRIDGE, J. H. (1988). Effect of acetylcholine on twitches, microscopic tension fluctuations and cooling contractures in rabbit ventricle. *Journal of Physiology* **404**, 53–69.
- BUCKELMANN, D. J., NABAUER, M. & ERDMANN, E. (1992). Intracellular calcium handling in isolated ventricular myocytes from patients with terminal heart failure. *Circulation* **85**, 1046–1055.
- BRIDGE, J. H. B. (1986). Relationships between sarcoplasmic reticulum and trans-sarcolemmal Ca transport revealed by rapidly cooling rabbit ventricular muscle. *Journal of General Physiology* **88**, 437–473.
- CONVERY, M. K. & HANCOX, J. C. (1998). Na^+-Ca^{2+} exchanger current measured in isolated rabbit ventricular myocytes compared using voltage ramp and step protocols. *Journal of Physiology* **509**, P. 155P.
- EISNER, D. A. (1990). Intracellular sodium in cardiac muscle: effects on contraction. *Quarterly Journal of Experimental Physiology* **75**, 437–457.
- GRAY, R. P., CAREY, P., SHERIDAN, D. J. & FRY, C. H. (1997). The relationship between intracellular $[Na]$, $[Na]_i$, and force-frequency response in hypertrophied guinea-pig left ventricular myocardium. *Journal of Physiology* **504**, P. 72P.
- GRYNKIEWICZ, G., POENIE, M. & TSIEN, R. Y. (1985). A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *Journal of Biological Chemistry* **260**, 3440–3450.
- HASENFUSS, G., MEYER, M., SCHILLINGER, W., PREUSS, M., PIESKE, B. & JUST, H. (1997). Calcium handling proteins in the failing human heart. *Basic Research in Cardiology* **92**, suppl. 1, 87–93.
- HE, H., GIORDANO, F. J., HILAL-DANDAN, R., CHOI, D., ROCKMAN, H. A., McDONOUGH, P. M., BLUHM, W. F., 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.
- HOBAI, I. A., BATES, J. A., HOWARTH, F. C. & LEVI, A. J. (1997). Inhibition by external Cd^{2+} of Na/Ca exchange and L-type Ca channel in rabbit ventricular myocytes. *American Journal of Physiology* **272**, H2164–2172.
- HOVE-MADSEN, L. & BERS, D. M. (1992). Indo-1 binding to protein in permeabilized ventricular myocytes alters its spectral and Ca binding properties. *Biophysical Journal* **63**, 89–97.
- HRYSKO, L. V., STIFFEL, V. & BERS, D. M. (1989). Rapid cooling contractures as an index of sarcoplasmic reticulum calcium content in rabbit ventricular myocytes. *American Journal of Physiology* **257**, H1369–1377.
- JANCZEWSKI, A. M., SPURGEON, H. A., STERN, M. D. & LAKATTA, E. G. (1995). Effects of sarcoplasmic reticulum Ca^{2+} load on the gain function of Ca^{2+} release by Ca^{2+} current in cardiac cells. *American Journal of Physiology* **268**, H916–920.
- KIMURA, J., MIYAMAE, S. & NOMA, A. (1987). Identification of sodium-calcium exchange current in single ventricular cells of guinea-pig. *Journal of Physiology* **384**, 199–222.
- KIMURA, J., NOMA, A. & IRISAWA, H. (1986). Na-Ca exchange current in mammalian heart cells. *Nature* **319**, 596–597.
- KURIHARA, S. & SAKAI, T. (1985). Effects of rapid cooling on mechanical and electrical responses in ventricular muscle of guinea-pig. *Journal of Physiology* **361**, 361–378.
- LEE, C. O. (1985). 200 years of digitalis: the emerging central role of the sodium ion in the control of cardiac force. *American Journal of Physiology* **249**, C367–378.
- NAQVI, R. U. & MACLEOD, K. T. (1994). Effect of hypertrophy on mechanisms of relaxation in isolated cardiac myocytes from guinea pig. *American Journal of Physiology* **267**, H1851–1861.
- NAQVI, R. U., TERRACCIANO, C. M. N. & MACLEOD, K. T. (1994). Rapid cooling of voltage-clamped guinea-pig cardiac myocytes produces an inward current which could be attributable to Na/Ca exchange. *Biophysical Journal* **66**, A96.
- NICOLL, D. A., LONGONI, S. & PHILIPSON, K. D. (1990). Molecular cloning and functional expression of the cardiac sarcolemmal Na^+-Ca^{2+} exchanger. *Science* **250**, 562–564.
- PIESKE, B., MAIER, L. S., MINAMI, K., JUST, H. & HASENFUSS, G. (1997). Frequency-dependent changes in intracellular Na concentration in isolated human myocardium. *Heart Failure* **97**, *European Society of Cardiology* **90** (abstract).
- SATOH, H., DELBRIDGE, L. M. D., BLATTER, L. A. & BERS, D. M. (1996). Surface: volume relationship in cardiac myocytes studied with confocal microscopy and membrane capacitance measurements: species-dependence and developmental effects. *Biophysical Journal* **70**, 1494–1504.

- SILVERMAN, H. S., HAIGNEY, M. C. P., GRIFFITHS, E. J., WEI, S., OCAMPO, C. J., PHILIPSON, K. D. & STERN, M. D. (1995). Excitation-contraction coupling in ventriculoocytes from transgenic mice with high level expression of canine sarcolemmal sodium-calcium exchange. *Circulation* **92**, I-236 (abstract).
- SIRI, F. M., KRUEGER, J., NORDIN, C., MING, Z. & ARONSON, R. S. (1991). Depressed intracellular calcium transients and contraction in myocytes from hypertrophied and failing guinea pig hearts. *American Journal of Physiology* **261**, H514–530.
- SITSAPESAN, R., MONTGOMERY, R. A. P., MACLEOD, K. T. & WILLIAMS, A. J. (1991). Sheep cardiac sarcoplasmic reticulum calcium-release channels: modification of conductance and gating by temperature. *Journal of Physiology* **434**, 469–487.
- SITSAPESAN, R. & WILLIAMS, A. J. (1997). Regulation of current flow through Ryanodine receptors by luminal Ca^{2+} . *Journal of Membrane Biology* **159**, 179–185.
- STEADMAN, B. W., MOORE, K. B., SPITZER, K. W. & BRIDGE, J. H. (1988). A video system for measuring motion in contracting heart cells. *IEEE Transactions on Biomedical Engineering* **35**, 264–272.
- STUDER, R., REINECKE, H., BILGER, J., ESCHENHAGEN, T., BÖHM, M., HASENFUSS, G., JUST, H., HOLTZ, J. & DREXLER, H. (1994). Gene expression of the cardiac Na^+-Ca^{2+} exchanger in end-stage human heart failure. *Circulation Research* **75**, 443–453.
- TERRACCIANO, C. M. N., NAQVI, R. U. & MACLEOD, K. T. (1995). Effects of rest interval on the release of calcium from the sarcoplasmic reticulum in isolated guinea pig ventricular myocytes. *Circulation Research* **77**, 354–360.
- TERRACCIANO, C. M. N. & MACLEOD, K. T. (1997a). Measurements of Ca entry and SR Ca content during the cardiac cycle in guinea-pig and rat ventricular myocytes. *Biophysical Journal* **72**, 1319–1326.
- TERRACCIANO, C. M. N. & MACLEOD, K. T. (1997b). Effects of lactate on the relative contribution of Ca^{2+} extrusion mechanisms to relaxation in guinea-pig ventricular myocytes. *Journal of Physiology* **500**, 557–570.
- TERRACCIANO, C. M. N., TWEEDIE, D. & MACLEOD, K. T. (1997). The effects of changes to action potential duration on the calcium content of the sarcoplasmic reticulum in isolated guinea-pig ventricular myocytes. *Pflügers Archiv* **443**, 542–544.
- VÁRRO, A., NEGRETTI, N., HESTER, S. B. & EISNER, D. A. (1993). An estimate of the calcium content of the sarcoplasmic reticulum in rat ventricular myocytes. *Pflügers Archiv* **423**, 158–160.
- WOLSKA, B. M. & SOLARO, R. J. (1996). Method for isolation of adult mouse cardiac myocytes for studies of contraction and microfluorimetry. *American Journal of Physiology* **271**, H1250–1255.
- YAO, A., SU, Z., NONAKA, A., ZUBAIR, I., LU, L., PHILIPSON, K. D., BRIDGE, J. H. B. & BARRY, W. H. (1998). The effects of overexpression of the Na^+/Ca^{2+} exchanger on $[Ca^{2+}]_i$ transients in murine ventricular myocytes. *Circulation Research* (in the Press).

Acknowledgements

We would like to thank Maren Koban for her invaluable help with the protein determination experiments. We also thank the British Heart Foundation and NIH (HL48509) for financial support.

Corresponding author

C. M. N. Terracciano: Imperial College School of Medicine, National Heart and Lung Institute, Dovehouse Street, London SW3 6LY, UK.

Email: c.terracciano@ic.ac.uk