Relaxation in rabbit and rat cardiac cells: species-dependent differences in cellular mechanisms

José W. M. Bassani, Rosana A. Bassani and Donald M. Bers

Department of Physiology, Loyola University School of Medicine,
Maywood, IL 60153, USA

1. The roles of the sarcoplasmic reticulum (SR) Ca^{2+}-ATPase and Na^+–Ca^{2+} exchange in Ca^{2+} removal from cytosol were compared in isolated rabbit and rat ventricular myocytes during caffeine contractures and electrically stimulated twitches. Cell shortening and intracellular calcium concentration ($[Ca^{2+}]_i$) were measured in indo-1-loaded cells.
2. Na^+–Ca^{2+} exchange was inhibited by replacement of external Na^+ by Li^+. To avoid net changes in cell or SR Ca^{2+} load during a twitch in 0 Na^+ solution, intracellular Na^+ (Na_i^+) was depleted using a long pre-perfusion with 0 Na^+, 0 Ca^{2+} solution. SR Ca^{2+} accumulation was inhibited by caffeine or thapsigargin (TG).
3. Relaxation of steady-state twitches was 2-fold faster in rat than in rabbit (before and after Na_i^+ depletion). In contrast, caffeine contractures (where SR Ca^{2+} accumulation is inhibited), relaxed faster in rabbit cells. Removal of external Na^+ increased the half-time for relaxation of caffeine contractures 15- and 5-fold in rabbit and rat myocytes respectively (and increased contracture amplitude in rabbit cells only). The time course of relaxation in 0 Na^+, 0 Ca^{2+} solution was similar in the two species.
4. Inhibition of the Na^+–Ca^{2+} exchange during a twitch increased the $[Ca^{2+}]_i$ transient amplitude ($\Delta[Ca^{2+}]_i$) by 50 % and the time constant of $[Ca^{2+}]_i$ decline (τ) by 45 % in rabbit myocytes. A smaller increase in τ (20 %) and no change in $\Delta[Ca^{2+}]_i$ were observed in rat cells in 0 Na^+ solution. $[Ca^{2+}]_i$ transients remained more rapid in rat cells.
5. Inhibition of the SR Ca^{2+}-ATPase during a twitch enhanced $\Delta[Ca^{2+}]_i$ by 25 % in both species. The increase in τ after TG exposure was greater in rat (9-fold) than in rabbit myocytes (2-fold), which caused $[Ca^{2+}]_i$ decline to be 70 % slower in rat compared with rabbit cells. The time course of $[Ca^{2+}]_i$ decline during twitch in TG-treated cells was similar to that during caffeine application in control cells.
6. Combined inhibition of these Ca^{2+} transport systems markedly slowed the time course of $[Ca^{2+}]_i$ decline, so that τ was virtually the same in both species and comparable to that during caffeine application in 0 Na^+, 0 Ca^{2+} solution. Thus, the combined participation of slow Ca^{2+} transport mechanisms (mitochondrial Ca^{2+} uptake and sarcolemmal Ca^{2+}-ATPase) is similar in these species.
7. We conclude that during the decline of the $[Ca^{2+}]_i$ transient, the Na^+–Ca^{2+} exchange is about 2- to 3-fold faster in rabbit than in rat, whereas the SR Ca^{2+}-ATPase is 2- to 3-fold faster in the rat. While the SR Ca^{2+}-ATPase is more powerful than the Na^+–Ca^{2+} exchange in both cell types the dominance is much more marked in rat (~13-fold *vs.* 2·5-fold in rabbit). Finally we estimate that the fraction of Ca^{2+} transported by the SR, Na^+–Ca^{2+} exchange and slow systems during a twitch are 70, 28 and 2 % respectively in rabbit myocytes and 92, 7 and 1 % respectively in rat myocytes.

It is generally accepted that excitation–contraction coupling in mammalian cardiac muscle involves Ca^{2+} release from the sarcoplasmic reticulum (SR) induced by Ca^{2+} influx through voltage-dependent sarcolemmal Ca^{2+} channels and possibly also via Na^+–Ca^{2+} exchange (Fabiato, 1985; Beuckelmann & Wier, 1988; Näbauer, Callewaert, Cleeman & Morad, 1989; Leblanc & Hume, 1990). Thus, Ca^{2+} for contraction activation originates from both the SR and the extracellular medium (for review, see Bers, 1991; Wier, 1990).

However, the relative participation of these sources of Ca^{2+} in activating contraction has been shown to differ among species. For instance, although the SR is considered the major source of Ca^{2+} for contraction, electrically evoked twitches in rabbit and guinea-pig ventricle are relatively little affected by treatment with ryanodine or caffeine (which inhibits SR function). The converse is observed in rat ventricle, where twitches are inhibited by up to 90% when the SR is inhibited (Sutko & Willerson, 1980; Bers, 1985; Mitchell, Powell, Terrar & Twist, 1987; Horackova, 1989; Lewartowski, Hansford, Langer & Lakatta, 1990). Similarly, treatment with thapsigargin (TG, which selectively inhibits the SR Ca^{2+}-ATPase) depresses the steady-state twitch and the accompanying $[Ca^{2+}]_i$ transient more markedly in rat (80–90%, Kirby, Sagara, Gaa, Inesi, Lederer & Rogers, 1992) than in rabbit and guinea-pig isolated ventricular myocytes (30–50%, Bassani, Bassani & Bers, 1993*a*; Lewartowski & Wolska, 1993). Moreover, the SR Ca^{2+}-induced Ca^{2+} release mechanism in hearts from adult rats is more developed than in those from adult rabbits (Fabiato, 1982).

The $[Ca^{2+}]_i$ decline and relaxation in rabbit and rat cardiac preparations depend primarily on SR Ca^{2+} uptake and on Ca^{2+} extrusion via Na^+–Ca^{2+} exchange (Bers & Bridge, 1989; Bers, Lederer & Berlin, 1990; O'Neill, Valdeolmillos, Lamont, Donoso & Eisner, 1991; Bassani, Bassani & Bers, 1992*a*). It is possible that trans-sarcolemmal Ca^{2+} movements are quantitatively more important during relaxation in rabbit than in rat (as discussed above for activation and proposed by Bers, 1985). That is, although SR Ca^{2+} uptake and release may normally dominate Ca^{2+} fluxes in both species (Bers, 1991), in rat cells the SR participation in relaxation may be greater than in rabbit cells. Similarly, Ca^{2+} extrusion via Na^+–Ca^{2+} exchange could be more important in rabbit than in rat ventricle during steady-state twitches.

In the present study, we investigated this possibility, analysing the participation of Na^+–Ca^{2+} exchange and the SR Ca^{2+} pump during relaxation and the decline of $[Ca^{2+}]_i$ in rabbit and rat ventricular myocytes. An important point in the present experiments is that the effects of inhibition of these Ca^{2+} transport systems were assessed during electrically stimulated twitches in intact cells, thus allowing us to compare rabbit and rat myocytes in near-physiological conditions. A fundamental difficulty in resolving the different contributions of Na^+–Ca^{2+} exchange and SR Ca^{2+}-ATPase to the normal twitch is that it is difficult to completely and selectively inhibit one system or the other without altering the SR Ca^{2+} content or the excitation–contraction coupling process. We have devised a procedure which overcomes this limitation using TG to inhibit the SR Ca^{2+} pump and removal of extracellular Na^+ to inhibit Na^+–Ca^{2+} exchange. Prior to perfusion with 0 Na^+ solution these cells were depleted of intracellular sodium (Na_i^+) to prevent Ca^{2+} influx via Na^+–Ca^{2+} exchange when extracellular Na^+ is removed. This allows us to study 'normal' twitches with either the Na^+–Ca^{2+} exchange or the SR Ca^{2+} pump selectively blocked. Our results reveal the existence of species differences in the participation of these mechanisms in Ca^{2+} removal from the cytosol.

METHODS

Cardiac myocyte isolation

The procedure for isolation of ventricular myocytes was previously described (Hryshko, Stiffel & Bers, 1989; Bassani *et al.* 1992*a*). Briefly, hearts were excised from adult male New Zealand White rabbits (1·5–2·5 kg) and Sprague–Dawley rats (300–350 g) anaesthetized with pentobarbitone sodium (rabbits, 70 mg kg^{-1}, I.V.; rats, 180 mg kg^{-1}, I.P.). The hearts were mounted in a Langendorff perfusion apparatus and perfused with nominally Ca^{2+}-free Tyrode solution for 6 min at 37 °C (flow rate of 20 ml min^{-1} for rabbit and 12 ml min^{-1} for rat). Perfusion was then switched to the same solution containing 1 mg ml^{-1} collagenase (Type B, Boehringer Mannheim) and 0·16 mg ml^{-1} pronase (Boehringer Mannheim). Perfusion continued until the heart became flaccid ($\sim$20–40 min). Then the ventricular tissue was dispersed and filtered. The cell suspension was rinsed several times, with a gradual increase in the Ca^{2+} concentration up to 1 mM (rat) or 2 mM (rabbit). The cells were plated on Plexiglass superfusion chambers (0·15 ml) in which the bottoms were formed by glass coverslips treated with laminin (Gibco, Grand Island, NY, USA) to enhance cell adhesion.

Cell shortening and fluorescence measurements

The chamber was placed on an inverted microscope (Nikon Diaphot, Tokyo, Japan) adapted for epifluorescence measurements. The cells were superfused with normal Tyrode solution at room temperature (22–23 °C) and field stimulated (square waves, amplitude 20–50% above threshold, 0·5 Hz) through a pair of platinum electrodes. Cell shortening was measured at both ends using a video-edge detection system (Crescent Electronics, Sandy, UT, USA).

For the fluorescence measurements, the cells were loaded with indo-1 by incubation with the acetoxymethyl ester form of the dye (indo-1 AM, 10 μM; Molecular Probes Inc., Eugene, OR, USA) for 15 min at room temperature. After loading, the cells were superfused with normal Tyrode solution for at least 40 min, to allow the wash-out of the extracellular dye and de-esterification of the intracellular indo-1. Electrically stimulated contractions were not significantly affected by the loading procedure.

The instrumentation for cell fluorescence measurement is described elsewhere (Bassani *et al.* 1992*a*, 1993*b*). The excitation wavelength was 365 nm and fluorescence emitted by the cell was recorded at 405 and 485 nm. The field illumination was restricted to a circular spot of 30 μm diameter. The microscope emission field was restricted to a single cell with the aid of an adjustable window. The background fluorescence recorded from a field of the same size at both wavelengths was subtracted from the signal recorded from the cell before the fluorescence ratio (405/485) was calculated. Measurements were not corrected for intracellular indo-1 binding and compartmentalization or cell autofluorescence. Fluorescence experiments were carried out under yellow light to minimize optical interference and indo-1 photobleaching.

Fluorescence ratios ($R = F_{405}/F_{485}$) were converted to free cytosolic Ca^{2+} concentrations according to the equation (Grynkiewicz, Poenie & Tsien, 1985):

$$[\mathrm{Ca}^{2+}]_i = K_d\beta\,[(R - R_{\min})/(R_{\max} - R)],$$

assuming a dissociation constant (K_d) of 250 nM (Grynkiewicz *et al.* 1985), which is very close to the value that we obtained *in vitro* (~240 nM). The β-value (ratio of the free to bound indo-1 fluorescence at 485 nm) was 3·0. The minimum and maximum values of R ($R_{\min}$ and $R_{\max}$) were determined *in vivo* in indo-1 AM-loaded cells superfused with solutions containing 5 mM ethyleneglycol-bis(β-aminoethylether)-*N*,*N*,*N'*,*N'*-tetraacetic acid (EGTA)/nominally zero Ca^{2+} and 2 mM Ca^{2+}, respectively, in the presence of the non-fluorescent Ca^{2+} ionophore BrA-23187 (10 μM, Calbiochem, La Jolla, CA, USA). For 20 min before and during $R_{\min}$ and $R_{\max}$ determination, the cells were treated with 3 μM carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone (FCCP, Sigma) and 10 mM 2-deoxyglucose (added to the glucose-free solution) to achieve metabolic inhibition and to limit hypercontracture upon the introduction of the high Ca^{2+} concentration. The values of $R_{\min}$ obtained under our conditions were 0·18 ± 0·02 ($n = 13$) and 0·17 ± 0·02 ($n = 14$) for rabbit and rat cells, respectively. $R_{\max}$ values were 0·54 ± 0·03 ($n = 13$) and 0·55 ± 0·04 ($n = 14$) in rabbit and rat, respectively.

Solutions

The solution for $R_{\min}$ and $R_{\max}$ determination contained (mM): 10 NaCl, 130 KCl, 1 MgCl$_2$ and 5 *N*-2-hydroxyethylpiperazine-*N'*-2-ethanosulphonic acid (Hepes). To this solution, 2 mM CaCl$_2$ or 5 mM EGTA was added and the pH adjusted to 7·2 at 22 °C. The control Tyrode solution had the following composition (mM): 140 NaCl, 6 KCl, 1 MgCl$_2$, 2 CaCl$_2$, 10 glucose and 5 Hepes. When rat myocytes were used, the CaCl$_2$ concentration was lowered to 1 mM in order to decrease spontaneous contractile activity (Capogrossi, Kort, Spurgeon & Lakatta, 1986). In the 0 Na$^+$, 0 Ca^{2+} solution, CaCl$_2$ was omitted, 1 mM EGTA was added and NaCl was replaced with LiCl. Also in the 0 Na$^+$ solution, LiCl substituted for NaCl, keeping CaCl$_2$ at 1 or 2 mM, depending on the species.

Thapsigargin (Calbiochem) stock solution was prepared in dimethyl sulphoxide. Dilution from stock solutions (2000-fold) was performed immediately before use. Caffeine was added as a solid. The pH of all solutions was adjusted to 7·4 at 22 °C.

Experimental procedure

Cells were perfused with control solution and stimulated at 0·5 Hz until twitch stabilization before each protocol. The electrical stimulation was interrupted and the cell perfused with 0 Na$^+$, 0 Ca^{2+} solution for 5–7 min. This procedure, while preventing SR Ca^{2+} loss during the rest (Bers, Bridge & Spitzer, 1989; Bassani *et al.* 1992*b*; Bers, Bassani, Bassani, Baudet & Hryshko, 1993; Bassani & Bers, 1993), allows Na^+_i depletion so that the subsequent reintroduction of Ca^{2+} only in the perfusion solution will not result in Ca^{2+} entry via Na$^+$–Ca^{2+} exchange (Bassani *et al.* 1992*a*, 1993*b*). The perfusion solution was then switched to either control or 0 Na$^+$ solution (containing Ca^{2+}) and an electrical stimulus was applied 10 s later. In some experiments, the cells were treated with 2·5 μM thapsigargin (TG) for the last 2 min of the period of pre-perfusion with 0 Na$^+$, 0 Ca^{2+} solution (Bassani *et al.* 1993*a*). It was not possible to perform all protocols on the same cell, due to run-down of the preparation during these long experiments or treatment with irreversible blockers, such as TG. However, all data from experiments using 0 Na$^+$ solution and/or TG were compared with those obtained in the same cell with control solution.

Caffeine (10 mM) was dissolved in control or 0 Na$^+$, 0 Ca^{2+} solution (whichever was the last solution to bathe the cell) and was rapidly switched on with the aid of a special switching device (Bassani *et al.* 1992*a*).

Action potential measurement

The same basic experimental protocol as above was used for action potential recording. Membrane potentials were recorded under whole-cell configuration using an Axopatch 1C patch clamp amplifier (Axon Instruments, Burlingame, CA, USA). Electrodes (borosilicate glass, 8–15 MΩ) were filled with the following solution (mM): 140 KCl, 7 NaCl, 10 Hepes and 0·5 EGTA, pH 7·2 at 22 °C. After formation of a gigaseal and rupture of the membrane patch, cells, under current-clamp mode, were stimulated by injection of suprathreshold current pulses. Action potentials were recorded in both control and 0 Na$^+$ solutions for each cell. Data were filtered at 10 kHz and analysed using pClamp software (Axon Instruments).

Statistical analysis

Data are presented as means ± S.E.M. Student's paired *t* test or two-way analysis of variance for paired measurements (followed by the Student–Newman–Keuls test for multiple comparisons) was used, when appropriate. Values of $P \leqslant 0.05$ were considered as statistically significant. Determination of the time constant (τ) of [Ca^{2+}]$_i$ decline of [Ca^{2+}]$_i$ transients was done by fitting the declining phase of the [Ca^{2+}]$_i$ transient to a monoexponential curve. For analysis of mechanical relaxation, however, half-times ($t_{1/2}$) were determined, since the data were not well described by a single exponential.

RESULTS

Caffeine-induced contractures in rabbit and rat myocytes

Amplitude of twitches and caffeine contractures

Figures 1 and 2 show twitches and caffeine contractures under steady-state conditions, i.e. they were obtained within a few seconds after interruption of electrical stimulation (see Methods). In this case, the SR Ca^{2+} content is expected to be the same for a twitch or a caffeine contracture. Differences in shortening amplitude (or [Ca^{2+}]$_i$ transient peak) would mostly reflect different degrees of SR Ca^{2+} release or the influence of Ca^{2+} transport systems.

The amplitude of the contracture induced by caffeine in control solution was significantly larger than that of a steady-state twitch at 0·5 Hz in ventricular myocytes isolated from hearts of the two species studied ($P < 0.05$, see Table 1). The amplitude of [Ca^{2+}]$_i$ transients (Δ[Ca^{2+}]$_i$) during caffeine application were also larger than during the steady-state twitch (Fig. 1 and Table 2). This difference is probably due to a combination of a larger fractional SR Ca^{2+} release than during a twitch and the prevention of SR Ca^{2+} accumulation by caffeine. Thus, Ca^{2+} re-uptake by the SR can limit the peak of the [Ca^{2+}]$_i$ transient during an electrically evoked contraction (Bassani *et al.* 1993*a*). This effect appears to be more pronounced in the rat, where the ratio of the caffeine contracture to twitch amplitude was higher in rats than in rabbits (2·1 and 1·4 respectively, and the same was observed for the $[Ca^{2+}]_i$ transients; see Tables 1 and 2).

Caffeine contractures in 0 Na^+, 0 Ca^{2+} solution were typically larger than in control solution, but the difference was significant only in rabbit cells ($P < 0·05$, see Tables 1 and 2, and Fig. 1). The larger caffeine contracture in 0 Na^+, 0 Ca^{2+} in rabbit myocytes probably indicates that the Na^+, Ca^{2+} exchange can curtail the caffeine contracture (and $[Ca^{2+}]_i$ transient) in rabbit ventricular myocytes in the same way that the SR Ca^{2+} pump curtails the twitch amplitude in both species. The smaller effect of Na^+ removal observed in rat myocytes might indicate that the Na^+–Ca^{2+} exchange is less able to curtail the peak of the contracture in this species than in the rabbit.

Time course of relaxation

When the time courses of relaxation of twitches and caffeine contractures were analysed, striking interspecies differences were observed. The half-times ($t_{½}$) of relaxation of twitches and caffeine contractures are presented in Table 1. Time constants of $[Ca^{2+}]_i$ decline are shown in Table 3. Steady-state twitches at 0·5 Hz relaxed much faster ($P < 0·001$) in rat myocytes than in rabbit cells (see Fig. 2*A*). The same difference was also observed for $[Ca^{2+}]_i$ transients (Fig. 1 and Table 3).

Caffeine-induced $[Ca^{2+}]_i$ transients and contractures declined slower than twitches, since SR Ca^{2+} uptake was prevented. In rabbit cells, the $t_{½}$ of relaxation was increased 3·3-fold compared with the twitch. However, in rat myocytes, the $t_{½}$ of relaxation of the caffeine contracture

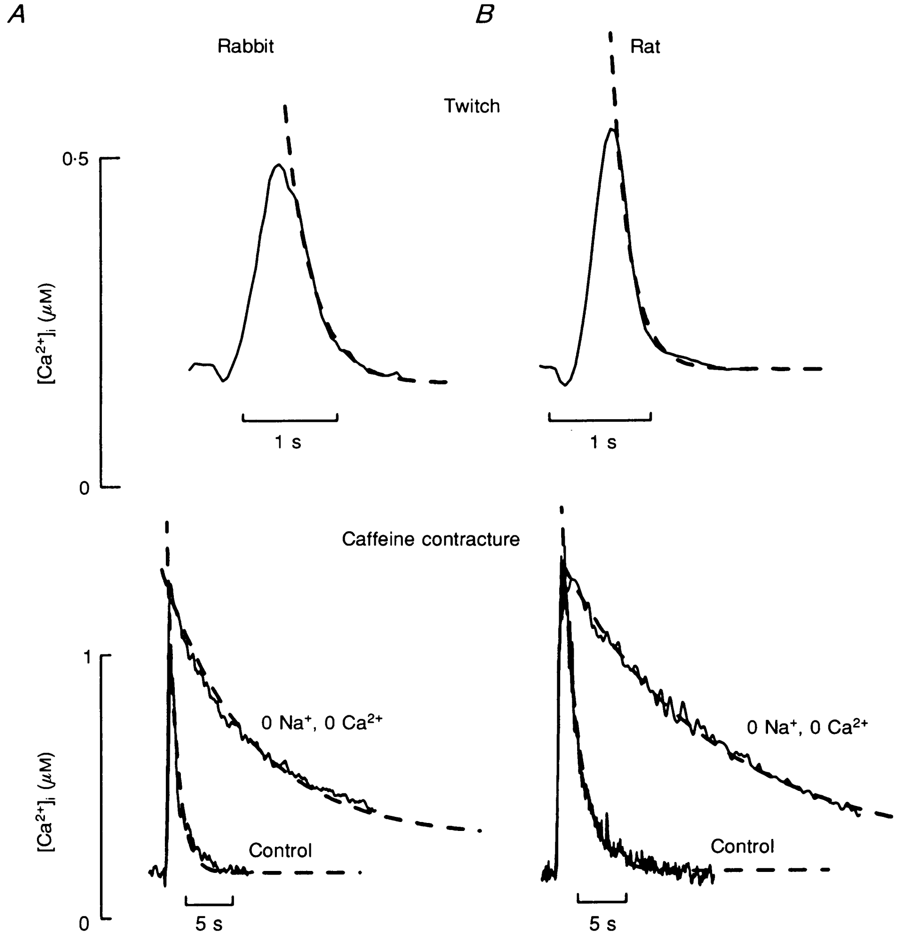

Figure 1. $[Ca^{2+}]_i$ transients in rabbit and rat myocytes

$[Ca^{2+}]_i$ transients obtained during steady-state twitches (upper panels) and during caffeine contractures (lower panels) in control and 0 Na^+, 0 Ca^{2+} solutions in one ventricular myocyte isolated from rabbit heart (*A*) and one from rat heart (*B*). Dashed lines represent exponential curves fitted to the decline of $[Ca^{2+}]_i$ ($R > 0·97$). In the cells shown, the time constant, τ for rabbit and rat respectively was 0·29 and 0·18 s for twitch, 1·0 and 1·73 s for control caffeine, and 10·4 and 13·0 s for caffeine, 0 Na^+, 0 Ca^{2+} (see Table 3 for pooled data).

Table 1. Amplitude and half-times ($t_{½}$) of relaxation of steady-state twitches (0·5 Hz) and caffeine contractures in control solution or 0 Na^+, 0 Ca^{2+} Tyrode solution determined in cardiac myocytes isolated from rabbit and rat hearts

	Amplitude (% of resting length)		$t_{½}$ of relaxation (s)	
	Rabbit	Rat	Rabbit	Rat
Twitch	22·57 ± 1·97 (12)	15·05 ± 1·66 (13)	0·18 ± 0·01 (12)	0·08 ± 0·01 (13)
Caffeine contracture				
Control	31·89 ± 3·03 (12)*	31·96 ± 2·99 (13)*	0·60 ± 0·09 (12)*	2·09 ± 0·29 (13)*
0 Na^+, 0 Ca^{2+}	41·39 ± 3·24 (12)* †	36·07 ± 2·69 (13)*	8·83 ± 0·45 (12)* †	10·16 ± 0·97 (13)* †

Means ± S.E.M. are presented, with the number of experiments in parentheses. Statistical comparisons between species are presented in the text. * Statistically significant difference from the control twitch; † statistically significant difference from the caffeine contracture in control solution ($P < 0·05$).

was 26-fold greater than that of the twitch. Figure 2*B* shows that caffeine contracture in rat cells relaxes significantly more slowly ($P < 0·001$) than in rabbit cells (see also Tables 1 and 3). Thus, it seems that inhibition of SR accumulation impairs relaxation more dramatically in rat cells.

Previous studies have suggested that the SR Ca^{2+} pump and Na^+–Ca^{2+} exchange are the primary systems involved in removing Ca^{2+} from the cytoplasm in cardiac muscle (Bers & Bridge, 1989; Hryshko *et al.* 1989; Crespo, Grantham & Cannell, 1990; Bassani *et al.* 1992*a*; Negretti, O'Neill & Eisner, 1993). Thus, when both of these systems are blocked during a caffeine contracture in 0 Na^+, 0 Ca^{2+} solution relaxation is expected to be very slow. Despite the differences in twitch and caffeine contractures between rabbit and rat, the time courses of $[Ca^{2+}]_i$ decrease and relaxation of caffeine contractures in 0 Na^+, 0 Ca^{2+} are remarkably similar (Fig. 2*C*, Tables 1 and 3). This result seems to indicate that the participation of slower Ca^{2+} transport systems (e.g. mitochondrial Ca^{2+} uptake and sarcolemmal Ca^{2+}-ATPase, see Bassani *et al.* 1992*a*) in

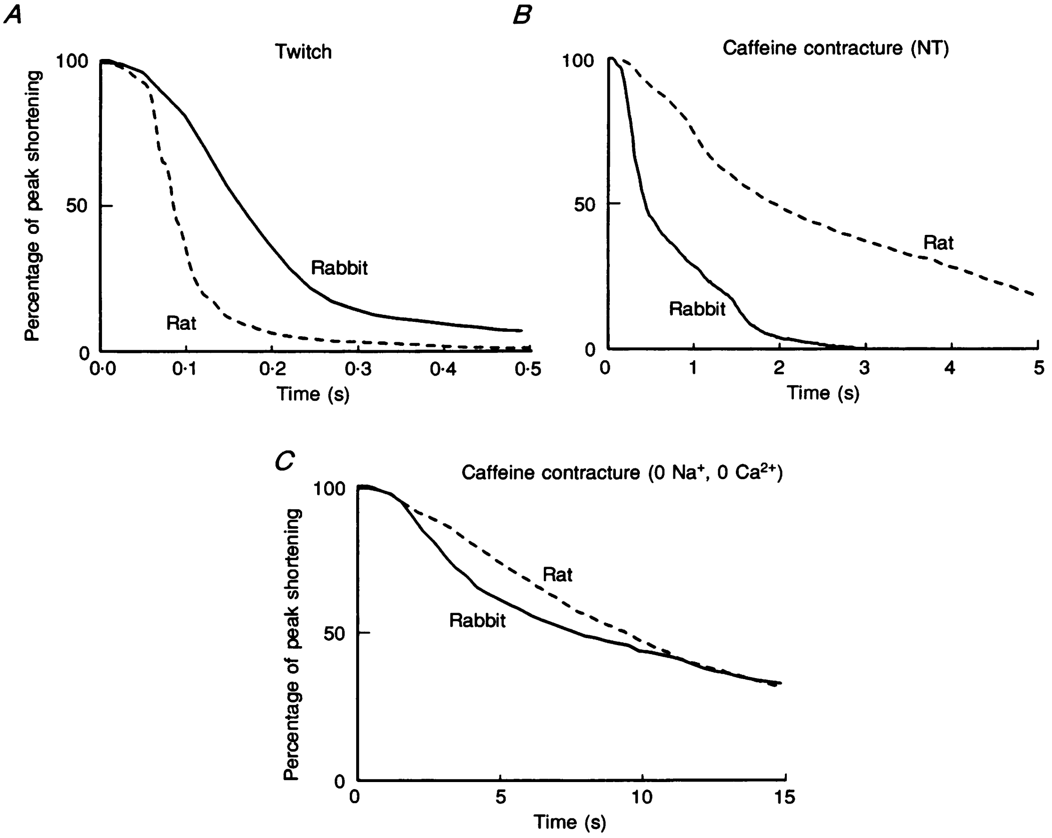

Figure 2. Relaxation in rabbit and rat myocytes

A, normalized mechanical relaxation of steady-state twitches. *B* and *C*, normalized mechanical relaxation of caffeine contractures in control (*B*) and 0 Na^+, 0 Ca^{2+} (*C*) solutions recorded in rat and rabbit ventricular myocytes.

Table 2. $\Delta[Ca^{2+}]_i$ (nM) during the peak of $[Ca^{2+}]_i$ transients evoked by electrical stimulation or rapid application of 10 mM caffeine to isolated rabbit and rat ventricular myocytes

	Rabbit	Rat
Twitches		
Steady-state twitch	382 ± 40 (13)	298 ± 13 (13)
Twitch after Na^+ depletion	327 ± 26 (13)	280 ± 14 (13)
Control	328 ± 28 (12)	296 ± 14 (10)
0 Na^+	484 ± 47 (12)*	295 ± 22 (10)
Control	308 ± 20 (4)	236 ± 15 (4)
TG	388 ± 59 (4)*	290 ± 22 (4)*
Control	476 ± 42 (2)	291 ± 43 (2)
0 Na^+	700 ± 18 (2)*	302 ± 42 (2)
0 Na^+ + TG	668 ± 33 (2)*	777 ± 216 (2)*
Caffeine-induced contractures		
Control	714 ± 103 (7)	1045 ± 99 (6)
0 Na, 0 Ca^{2+}	1042 ± 104 (7)†	1110 ± 130 (6)

Different experimental conditions, such as absence of Na^+ and/or Ca^{2+} in the perfusate and pretreatment with TG, are indicated. Means ± S.E.M. are presented, with the number of experiments in parentheses. Statistical comparisons between species are presented in the text. * Statistically significant difference from the control twitch; † statistically significant difference from the caffeine contracture in control solution ($P < 0.05$).

relaxation is similar in both species. The 15-fold slowing of relaxation in rabbit cells when Na^+–Ca^{2+} exchange was inhibited (compared to only ~5 times in the rat) suggests a greater participation of Na^+–Ca^{2+} exchange in $[Ca^{2+}]_i$ removal in rabbit than in rat ventricular myocytes. This is consistent with the faster decline of caffeine contractures in rabbit cells when Na^+–Ca^{2+} exchange was primarily responsible (Fig. 2*B*).

In contrast, the results with rat myocytes would suggest a lesser role for the Na^+–Ca^{2+} exchange in relaxation. The faster twitch relaxation (despite a slower caffeine contracture) in rat cells might indicate that the SR in rat myocytes can take up Ca^{2+} faster than that in rabbit cells. However, this comparison does not allow us to make clear inferences about the relative participations of the SR Ca^{2+}-ATPase and the Na^+–Ca^{2+} exchange in the relaxation of a normal twitch, during which both systems are simultaneously functioning. Moreover, the different time course of the action potential in rabbit and rat ventricle (e.g. Shattock & Bers, 1987) might modulate the operation of the membrane potential-sensitive Na^+–Ca^{2+} exchange in a species-dependent fashion (Bridge, Spitzer & Ershler, 1988; Bers & Bridge, 1989). Therefore, we also analysed the effects of individual and combined inhibition of the SR Ca^{2+}-ATPase and Na^+–Ca^{2+} exchange during a more physiological event, the electrically evoked twitch.

Table 3. Time constants of $[Ca^{2+}]_i$ decline (τ) during $[Ca^{2+}]_i$ transients evoked by electrical stimulation or rapid application of 10 mM caffeine to isolated rabbit and rat ventricular myocytes

	Rabbit	Rat
Twitches		
Steady-state twitch	0·505 ± 0·077 (13)	0·194 ± 0·010 (13)
Twitch after Na^+ depletion	0·407 ± 0·026 (13)	0·196 ± 0·012 (13)
Control	0·406 ± 0·028 (12)	0·198 ± 0·015 (10)
0 Na^+	0·588 ± 0·054 (12)*	0·245 ± 0·017 (10)
Control	0·496 ± 0·034 (4)	0·181 ± 0·008 (4)
TG	0·978 ± 0·120 (4)*	1·655 ± 0·296 (4)*
Control	0·373 ± 0·067 (2)	0·201 ± 0·023 (2)
0 Na^+	0·591 ± 0·017 (2)*	0·228 ± 0·043 (2)
0 Na^+ + TG	12·292 ± 1·597 (2)*	12·779 ± 0·448 (2)*
Caffeine-induced contractures		
Control	0·945 ± 0·101 (7)	1·596 ± 0·087 (6)
0 Na^+, 0 Ca^{2+}	12·329 ± 1·498 (7)†	12·093 ± 0·642 (6)†

Statistical comparisons between species are presented in the text. * Statistically significant difference from the control twitch; † statistically significant difference from the caffeine contracture in control solution ($P < 0.05$).

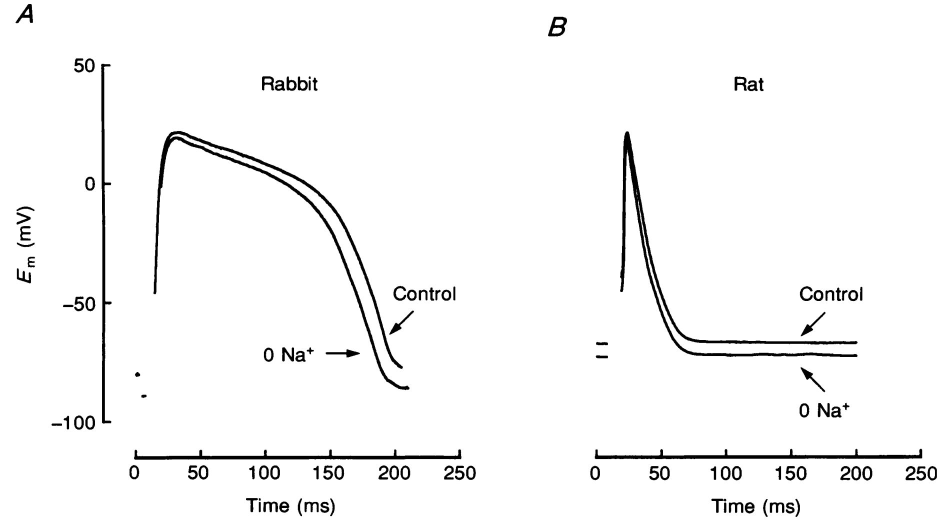

Figure 3. Action potentials in rabbit and rat myocytes
Action potentials measured under current-clamp mode in rabbit (*A*) and rat (*B*) ventricular myocytes in control and 0 Na^+ solution, in which NaCl was totally replaced by an equimolar amount of LiCl. Measurements were obtained after 5–7 min pre-perfusion with 0 Na^+, 0 Ca^{2+} solution.

$[Ca^{2+}]_i$ transients during electrically stimulated twitches with inhibition of Na^+–Ca^{2+} exchange and/or SR Ca^{2+}-ATPase

Figure 3 depicts action potentials recorded in rabbit and rat isolated ventricular myocytes. In both species, the cells developed contractions (not shown) accompanying the action potential. As can be seen in Fig. 3, control action potentials in rat myocytes were considerably shorter, lacking the plateau observed in rabbit cells. Total replacement of extracellular Na^+ by Li^+ led to slight membrane hyperpolarization (5–10 mV) and modestly shortened the action potential in both cell types. However, the difference between species in the time course of repolarization observed in control solution was maintained in 0 Na^+ solution.

Inhibition of the Na^+–Ca^{2+} exchange

Figure 4 shows $[Ca^{2+}]_i$ transients evoked by electrical stimulation in control and 0 Na^+ solution obtained in rabbit and rat ventricular myocytes. Values of the $Ca^{2+}{}_i$ transient amplitude ($\Delta[Ca^{2+}]_i$) and τ of $[Ca^{2+}]_i$ decline are presented in Tables 2 and 3, respectively.

Diastolic $[Ca^{2+}]_i$ during steady-state stimulation was not significantly different between species (208 ± 16 nM in rabbit and 195 ± 18 nM in rat, $n = 13$). However, $\Delta[Ca^{2+}]_i$ was higher in rabbit than in rat cells ($P < 0.05$, see Table 2). This may be partly due to the lower extracellular $[Ca^{2+}]$ ($[Ca^{2+}]_o$) used in the control solution for the rat (see Methods). The $[Ca^{2+}]_i$ transient was also significantly faster in rat cells (~2-fold, $P < 0.001$), in agreement with the difference observed for $t_{1/2}$ of the mechanical relaxation of

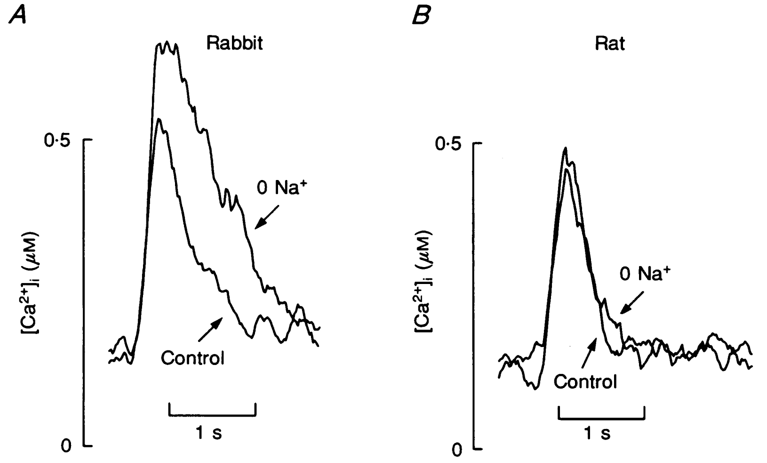

Figure 4. Inhibition of Na^+–Ca^{2+} exchange during a twitch
$[Ca^{2+}]_i$ transients recorded during electrically stimulated twitches in rabbit (*A*) and rat (*B*) ventricular myocytes. Stimulation was applied 10 s after switching to control or 0 Na^+ solution after 5–7 min pre-perfusion with 0 Na^+, 0 Ca^{2+} solution.

the steady-state twitch (see Tables 1 and 3). This difference was also present when rat cells were bathed with 2 mM $[\mathrm{Ca}^{2+}]_o$ (not shown).

The pre-perfusion period with 0 Na^+, 0 Ca^{2+} solution did not significantly change the $\Delta[\mathrm{Ca}^{2+}]_i$ or τ (see Tables 2 and 3), but resting $[\mathrm{Ca}^{2+}]_i$ was significantly decreased (to 149 ± 13 nM in rabbit and to 117 ± 10 nM in rat; $n = 13$, $P < 0{\cdot}01$ for both comparisons). Such a decline in free $[\mathrm{Ca}^{2+}]_i$ might be expected after prolonged perfusion with EGTA-containing solution. However, the absence of significant alteration in $\Delta[\mathrm{Ca}^{2+}]_i$ after the pre-perfusion is in agreement with preservation of SR Ca^{2+} content during rest in Na^+-free solution (Bers *et al.* 1989, 1993; Bassani *et al.* 1992*b*; Bassani & Bers, 1993).

To prevent Ca^{2+} entry via Na^+–Ca^{2+} exchange in these experiments with 0 Na^+, cells were depleted of Na_i^+ prior to twitches in control and 0 Na^+ solutions. After pre-perfusion with 0 Na^+, 0 Ca^{2+}, reintroduction of Ca_o^{2+}did not significantly increase basal $[\mathrm{Ca}^{2+}]_i$ (rabbit: 144 ± 13 nM and 160 ± 27 nM in control and 0 Na^+ solutions, respectively, $n = 12$; rat: 105 ± 10 nM and 106 ± 8 nM in control and 0 Na^+ solutions, respectively, $n = 10$). This shows that Ca^{2+} entry into the cell upon readmission of Ca^{2+} in the perfusate was prevented by the long pre-perfusion in 0 Na^+, 0 Ca^{2+}.

Removal of Na_o^+ in rabbit myocytes increased $\Delta[\mathrm{Ca}^{2+}]_i$ and τ by 45 % ($P < 0{\cdot}05$), whereas in rat cells τ was increased by only 20 % and this change was not statistically significant. An analysis of variance showed that the effects of inhibition of the Na^+–Ca^{2+} exchange on the $[\mathrm{Ca}^{2+}]_i$ transient were highly dependent on the cell type studied, considering either $\Delta[\mathrm{Ca}^{2+}]_i$ ($P < 0{\cdot}01$) or τ ($P < 0{\cdot}05$). The larger Ca_i^{2+}transients and greater slowing of relaxation in rabbit cells by blocking Na^+–Ca^{2+} exchange agrees with experiments with caffeine application above (see Fig. 1 and Table 1 and Bassani *et al.* 1992*a*). This result confirms that the participation of Na^+–Ca^{2+} exchange in the removal of Ca^{2+} from the cytoplasm is more prominent in rabbit than in rat ventricular cells.

Inhibition of the SR Ca^{2+}-ATPase

For these experiments it was important to have a normal SR Ca^{2+} load but complete inhibition of the SR Ca^{2+} pump at the test twitch. To achieve this specific condition, we applied TG for 2 min in 0 Na^+, 0 Ca^{2+} solution prior to the test contraction in control solution or 0 Na^+ solution (Bassani *et al.* 1993*a*). The efficacy of TG treatment was tested in parallel experiments and also *post hoc* in each cell after the test twitch by assessing the ability of the SR to be reloaded after depletion. If any SR reloading was detected the cell was discarded. Some increase in resting $[\mathrm{Ca}^{2+}]_i$ was observed after treatment with TG (from 167 ± 34 nM to 245 ± 67 nM in rabbit; and from 117 ± 17 nM to 162 ± 23 nM in rat; $n = 4$; see Fig. 5). This could be attributed to net Ca^{2+} leakage from the SR (due to SR Ca^{2+}-ATPase inhibition) and accumulation in the cytosol (due to inhibition of the Na^+–Ca^{2+} exchange during pre-perfusion with 0 Na^+, 0 Ca^{2+} solution). However, during 0 Na^+, 0 Ca^{2+} perfusion the loss of Ca^{2+} by the SR when TG was added appeared to be very small. Parallel experiments using caffeine-induced contractures rather than a twitch indicated that the $\Delta[\mathrm{Ca}^{2+}]_i$ in response to caffeine after TG exposure as described was more than 90 % of that obtained prior to TG treatment (see Bassani *et al.* 1993*a*). Longer incubations with TG gradually depleted the SR, even in 0 Na^+, 0 Ca^{2+} solution (see Discussion).

The effect of TG on $[\mathrm{Ca}^{2+}]_i$ transients during a twitch is shown in Fig. 5 and Tables 2 and 3. TG induced an increase in $\Delta[\mathrm{Ca}^{2+}]_i$ of ~25 % in both cell types, although the SR Ca^{2+} content was not increased. It may also be noted that if

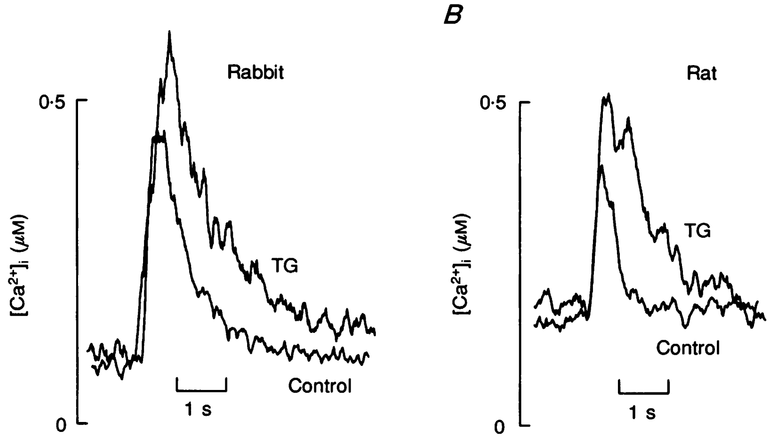

Figure 5. Inhibition of SR Ca^{2+}-ATPase during a twitch
$[\mathrm{Ca}^{2+}]_i$ transients recorded during electrically stimulated twitches in rabbit (*A*) and rat (*B*) ventricular myocytes before (control) and after treatment with 2·5 μM thapsigargin (TG). Stimulation was applied 10 s after switching to control solution after 5–7 min pre-perfusion with 0 Na^+, 0 Ca^{2+} solution. TG treatment (2 min exposure) was performed during a second pre-perfusion period.

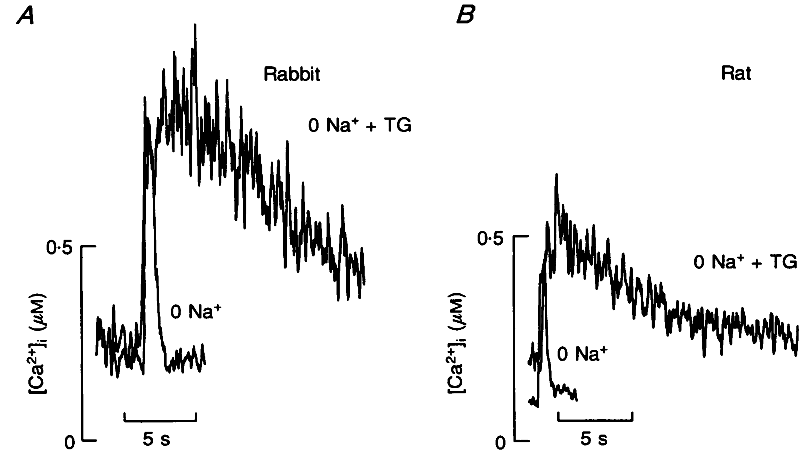

Figure 6.

$[Ca^{2+}]_i$ transients obtained during electrically stimulated twitches in rabbit (*A*) and rat (*B*) ventricular myocytes in 0 Na^+ solution before (0 Na^+) and after treatment with 2·5 μM thapsigargin (0 Na^+ + TG). In both cases, cells were pre-perfused with 0 Na^+, 0 Ca^{2+} solution for 5–7 min.

TG had significantly reduced SR Ca^{2+} content, larger twitches would not have been expected in Fig. 5 with TG. Thus, in both species, SR Ca^{2+} uptake by the SR seems to limit the peak of the $[Ca^{2+}]_i$ transient. This increase is much less than that observed when caffeine contractures are compared with twitches (see Table 2). This would be expected, since during a twitch only about half of the SR Ca^{2+} content is released (Bassani *et al.* 1993*a*), while continuous exposure to 10 mM caffeine empties the releasable Ca^{2+} pool in the SR (Bassani *et al.* 1993*b*).

Similarly, TG significantly prolonged the time course of $[Ca^{2+}]_i$ decline during the twitch in both species ($P < 0·001$). However, in this case the extent of the effects was species dependent ($P < 0·05$), with a 9-fold increase of τ in rat

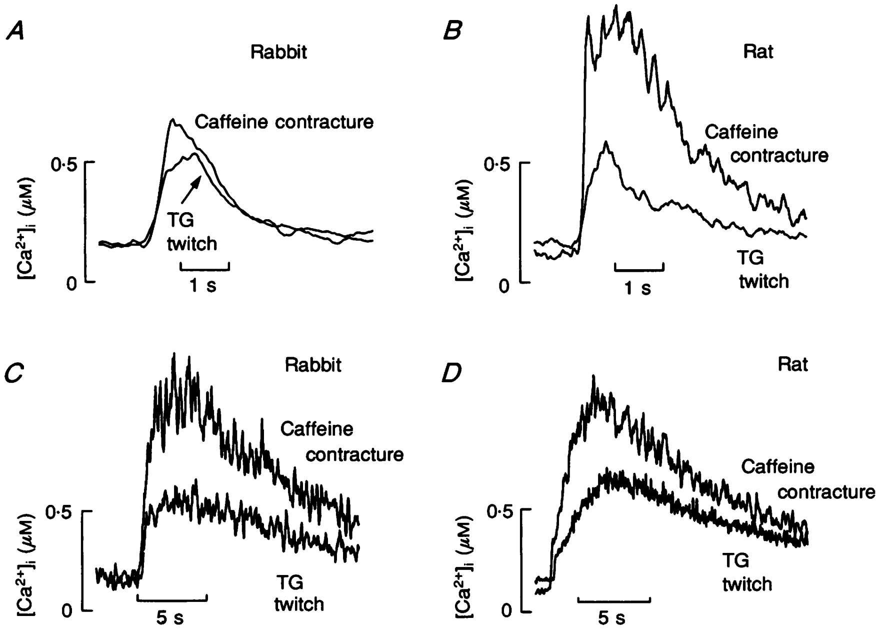

Figure 7. Superimposed $[Ca^{2+}]_i$ transients recorded during twitches with TG and caffeine-induced contractures

A and *B*, control solutions; *C* and *D*, 0 Na^+ solutions; *A* and *C*, experiments with rabbit; *B* and *D*, experiments with rat. $[Ca^{2+}]_i$ transients were obtained during electrical stimulation in control and 0 Na^+ solutions after thapsigargin treatment and also when SR Ca^{2+} uptake was inhibited during caffeine-induced contractions. Cells were pre-perfused with 0 Na^+, 0 Ca^{2+} solution for 5–7 min.

myocytes and only 2-fold in rabbit cells. After treatment of rat cells with TG, $[Ca^{2+}]_i$ decrease during the twitch (presumably due to Na^+–Ca^{2+} exchange) became significantly slower than in rabbit cells (70%, $P < 0{\cdot}05$). This indicates not only that the Na^+–Ca^{2+} exchange is slower in rat, but also that the SR Ca^{2+} uptake is faster in rat compared with rabbit myocytes.

Combined inhibition of Na^+–Ca^{2+} exchange and SR Ca^{2+}-ATPase

In some experiments, both fast Ca^{2+} transport systems were inhibited by evoking an electrically stimulated twitch in 0 Na^+ solution after treatment with TG. For better comparison, a twitch in 0 Na^+ solution was also obtained before exposure to TG. Figure 6 shows typical $[Ca^{2+}]_i$ traces with this protocol. Quantitative data are presented in Tables 2 and 3.

In rabbit myocytes, combining 0 Na^+ with TG produced no additional increase of $\Delta[Ca^{2+}]_i$ compared with 0 Na^+ alone. However, in rat cells, a substantial increase of $\Delta[Ca^{2+}]_i$ could be observed with addition of TG ($P < 0{\cdot}05$). This may indicate that the SR Ca^{2+} pump limits the peak $[Ca^{2+}]_i$ reached during a twitch more effectively in the rat.

In both species, the combined exposure to 0 Na^+ and TG had a dramatic effect on the time course of $[Ca^{2+}]_i$ decline ($P < 0{\cdot}01$ for τ values). This effect of TG when Na^+–Ca^{2+} exchange was already blocked was much greater than in control solution. Such a difference is not surprising since in control solution Na^+–Ca^{2+} exchange would partially compensate for the elimination of SR Ca^{2+} uptake, while in 0 Na^+ solution only slow Ca^{2+} transport mechanisms would be left to clear Ca^{2+} from the myoplasm (i.e. mitochondrial Ca^{2+} uptake and sarcolemmal Ca^{2+}-ATPase, Bassani *et al.* 1992*a*). Again, as observed for TG alone, the lengthening of $[Ca^{2+}]_i$ removal was more accentuated in rat (~65- and 55-fold with respect to control and 0 Na^+ solutions, respectively) than in rabbit ventricular cells (~30- and 20-fold, respectively).

Now, when both fast Ca^{2+} transport mechanisms were inhibited, the time course of $[Ca^{2+}]_i$ decline during the twitch was virtually the same in rabbit and rat myocytes ($\tau \approx 12$ s, see Table 3), something which had also been observed using caffeine in 0 Na^+, 0 Ca^{2+} solution (Fig. 2*C* and Table 3). Thus, this is one more indication that the combined slow mechanisms probably transport Ca^{2+} at similar rates in the two species, at least in the conditions of a twitch or caffeine induced contracture.

Figure 7 shows that although the $\Delta[Ca^{2+}]_i$ observed during caffeine application was higher than that during a twitch after TG treatment, in both conditions the $[Ca^{2+}]_i$ transients declined with a strikingly similar time course in rabbit and rat cells (see Table 3 and Fig. 7). This similarity for twitches in TG and caffeine application was observed for both Na^+-containing solution ($\tau \approx 0{\cdot}9$ s for rabbit and $1{\cdot}6$ s for rat) and Na^+-free solutions ($\tau \approx 12$ s for both).

DISCUSSION

During relaxation, cytosolic Ca^{2+} is both extruded from the cell and sequestered into intracellular stores. The predominant mechanism for Ca^{2+} extrusion is the Na^+–Ca^{2+} exchange, while the sarcolemmal Ca^{2+}-ATPase appears to make a minor contribution to this process. Similarly, although some Ca^{2+} may be sequestered by mitochondria, the major intracellular Ca^{2+} sink is the SR (Bers & Bridge, 1989; Bers *et al.* 1990; Crespo *et al.* 1990; O'Neill *et al.* 1991; Bassani *et al.* 1992*a*, 1993*b*; Negretti *et al.* 1993). In the present study, we observed different contributions of Ca^{2+} extrusion and intracellular Ca^{2+} sequestration in intact rat and rabbit isolated cardiac myocytes.

Techniques and assumptions

In the first part of this study, we analysed the decline of $[Ca^{2+}]_i$ transients and relaxation of the contracture induced by 10 mM caffeine. Because caffeine activates SR Ca^{2+}-release channels (Rousseau & Meissner, 1989), it strongly promotes SR Ca^{2+} release and by so doing, prevents Ca^{2+} accumulation by the SR. Thus, relaxation of the caffeine contracture depends mainly on Ca^{2+} transport by other systems (e.g. Na^+–Ca^{2+} exchange, mitochondria and sarcolemmal Ca^{2+}-ATPase: Bers & Bridge, 1989; O'Neill *et al.* 1991; Bassani *et al.* 1992*a*). While caffeine does have other complicating effects, such as inhibiting phosphodiesterases and increasing myofilament Ca^{2+} sensitivity (Wendt & Stephenson, 1983), caffeine-induced contractures and $[Ca^{2+}]_i$ transients can still be extremely useful. However, one cannot directly compare caffeine-induced contractures with twitches, partly because of these side effects. Additionally, during a twitch, only about half of the SR Ca^{2+} is released (Bassani *et al.* 1993*a*) and the change in membrane potential can modulate the Na^+–Ca^{2+} exchange. Thus, $[Ca^{2+}]_i$ transients during twitches were studied in more detail.

A unique aspect of the present study is that we have been able to inhibit selectively either the Na^+–Ca^{2+} exchanger or the SR Ca^{2+}-ATPase (or both) during an otherwise normal twitch contraction. During 5–7 min of quiescence in 0 Na^+, 0 Ca^{2+} solution in both rabbit and rat ventricular myocytes the SR Ca^{2+} content stays constant and $[Na^+]_i$ is decreased to levels where Ca^{2+} entry via Na^+–Ca^{2+} exchange is prevented upon readmission of $[Ca^{2+}]_o$ (Bassani *et al.* 1993*a*; Bassani & Bers, 1993; Bers *et al.* 1993). This was even the case when TG was added during the last 2–3 min of quiescence to inhibit the SR Ca^{2+}-ATPase. About 90 s is required for 2–5 μM TG to completely block the SR Ca^{2+}-ATPase (Bassani *et al.* 1993*a*). The protocol used here (test contractions induced 2–3 min after TG addition in 0 Na^+, 0 Ca^{2+} solution) was successful, since it allowed complete block of the SR Ca^{2+} pump without appreciable loss of SR Ca^{2+} content.

However, if too much time elapses between full blockade of the pump by TG and the test contraction, the SR Ca^{2+} content will slowly decline. Thus there is a relatively narrow window of time where these experiments can be done (in our case 2–3 min after TG addition). We also took advantage of the ability of Li^+ to substitute for Na^+ in carrying current through Na^+ channels during the action potential (but not Na^+–Ca^{2+} exchange). This allowed us to record near-normal action potentials in the complete absence of Na^+–Ca^{2+} exchange.

It is not obvious why replacement of Na^+ with Li^+ caused the modest hyperpolarization of the membrane potential. However, if it is due to a net outward current shift the slight shortening of action potential duration could be due to the same effect. The fact that the action potential alteration was modest when Na^+–Ca^{2+} exchange was abolished would suggest that this system is only a minor contributant to determining action potential configuration.

To inhibit the SR Ca^{2+}-ATPase, we used TG, which is able to prevent SR Ca^{2+} accumulation in cardiac cells without significantly affecting other ATPases, SR Ca^{2+} release or Ca^{2+} currents (Kirby *et al.* 1992; Wrzosek, Schneider, Grueninger & Chiesi, 1992; Bassani *et al.* 1993*a*; Lewartowski & Wolska, 1993). We analysed only the first twitch evoked after TG treatment, during which approximately half of the steady-state SR Ca^{2+} load is released (Bassani *et al.* 1993*a*). This fraction of SR Ca^{2+} release (assessed by comparing the $[Ca^{2+}]_i$ transient during a subsequent caffeine application with that evoked by caffeine before TG treatment) was not changed after the long pre-perfusion with 0 Na^+, 0 Ca^{2+} solution (not shown). Thus, it was possible to compare the peak of the $[Ca^{2+}]_i$ transient at a twitch with a control twitch obtained before TG treatment. Unfortunately, the irreversible nature of TG action precluded comparisons of repeated tests with TG in the same cell (e.g. direct comparison of TG effects in control *vs.* 0 Na^+ solution).

To inhibit Na^+–Ca^{2+} exchange during a twitch the cell was depleted of Na_i^+ by perfusion with 0 Na^+, 0 Ca^{2+} solution for 5–7 min prior to the test contraction. Thus a test twitch without Na^+–Ca^{2+} exchange was compared with a control twitch under the same conditions in control solution. A potential shortcoming of this procedure where Na_i^+ is depleted to very low levels prior to the test and control twitches could be the following. If appreciable Ca^{2+} influx via Na^+–Ca^{2+} exchange occurred during a normal twitch, this component would be lost. However, experiments in rabbit ventricle indicate that Ca^{2+} influx via Na^+–Ca^{2+} exchange is probably very small during a normal twitch unless the cell is Na^+ loaded (Bers, Christensen & Nguyen, 1988). Indeed, we find that the control $\Delta[Ca^{2+}]_i$ after Na_i^+ depletion is similar to the steady-state control (while a decrease in resting $[Ca^{2+}]_i$ was observed, there was no significant difference in $\Delta[Ca^{2+}]_i$ or τ). The lack of significant effect of $[Na^+]_i$ depletion on the control twitch and $[Ca^{2+}]_i$ transient indicates that Ca^{2+} influx via Na^+–Ca^{2+} exchange does not appear to contribute appreciably to the $[Ca^{2+}]_i$ transient (or trigger SR Ca^{2+} release) under control conditions in an intact myocyte (Bers *et al.* 1988; but see also Leblanc & Hume, 1990).

Slow relaxation without SR Ca^{2+}-ATPase or Na^+–Ca^{2+} exchange

When both the SR Ca^{2+}-ATPase and Na^+–Ca^{2+} exchange are inhibited the decline of the $[Ca^{2+}]_i$ transient was very slow ($\tau \approx 12$ s) and was the same for both rabbit and rat myocytes. These values were also the same whether SR Ca^{2+} accumulation was prevented by TG or caffeine (see Table 3). The slow decline of $[Ca^{2+}]_i$ in rabbit ventricular myocytes under these conditions is attributable to the combination of mitochondrial Ca^{2+} uptake and extrusion by the sarcolemmal Ca^{2+}-ATPase, with the two systems contributing about equally (Bassani *et al.* 1992*a*, 1993*b*). In the rat we have not carried out the same complete analysis of the relative contributions of mitochondrial Ca^{2+} uptake and sarcolemmal Ca^{2+}-ATPase. Thus, we can only conclude that the combined effects of these two systems appear to be the same in rat as in rabbit.

Na^+–Ca^{2+} exchange in relaxation

Relaxation of caffeine-induced contractures (and $[Ca^{2+}]_i$ transients) is faster in rabbit than in rat cells. Twitches with TG (and $[Ca^{2+}]_i$ transients) also relax faster in rabbit than in rat cells. With SR Ca^{2+} uptake blocked, the main mechanism for Ca^{2+} removal from the myoplasm is Na^+–Ca^{2+} exchange. Thus, these results suggest that the Na^+–Ca^{2+} exchange system is about 2- to 3-fold faster in rabbit than in rat. Since these results were obtained in cells where $[Na^+]_i$ was depleted, the lower Na^+–Ca^{2+} exchange activity cannot be attributed to a higher $[Na^+]_i$ in rat ventricle (Shattock & Bers, 1989).

This conclusion agrees with the 3–5 times more Na^+–Ca^{2+} exchange current measured in giant patches from rabbit than from rat myocytes (D. W. Hilgemann, personal communication) and much lower Na^+–Ca^{2+} exchange current in rat than in guinea-pig myocytes (Sham, Hatem & Morad, 1993). Moreover, Na^+-dependent Ca^{2+} transport in cardiac sarcolemmal vesicles has been reported to be lower in rat than in rabbit (Vetter, Kemsies & Schulze, 1987). Thus, it appears that the greater participation of Ca^{2+} extrusion by Na^+–Ca^{2+} exchange in rabbit than in rat myocytes is due to species-dependent difference in the intrinsic characteristics of this mechanism (e.g. possibly density of exchangers), rather than functional differences arising from modifications of the driving force.

The Na^+–Ca^{2+} exchanger is known to be sensitive to membrane potential (e.g. Bridge *et al.* 1988). Thus, it may be surprising that the τ values were the same for caffeine-induced contractures (at resting membrane potential) and twitches with TG (with accompanying action potentials) for rabbit and rat. This is probably because almost all of the declining phase of the $[Ca^{2+}]_i$ transient occurs after the action potential is over and thus our values of τ are probably relevant to resting membrane potentials (especially in rat). If we were to explore earlier times during the action potential, we would expect to see some effects of membrane potential. As suggested by Shattock & Bers (1989), it is possible that the early repolarization of the rat action potential stimulates more Ca^{2+} efflux via Na^+–Ca^{2+} exchange than would occur at plateau potentials (thus enhancing an intrinsically weaker exchanger). However, the Na^+–Ca^{2+} exchange still appears to play a relatively weak role in rat myocytes.

Furthermore since the Na^+–Ca^{2+} exchanger is slower in rat cells, the slow transport systems (mitochondrial and sarcolemmal Ca^{2+}-ATPase) may only be about 5–8 times slower than the Na^+–Ca^{2+} exchange in rat (*vs.* 12–20 times in rabbit, based on values in Tables 1 and 3). This may partially account for the finding that relaxation of caffeine-induced contractures in rat cells are less sensitive to membrane potential than similar experiments with rapid cooling contractures in rabbit (Bers & Bridge, 1989; O'Neill *et al.* 1991). That is, in rat myocytes the slow systems may compete better with the weaker Na^+–Ca^{2+} exchange.

Inhibition of Na^+–Ca^{2+} exchange even increases control twitch amplitude, $\Delta[Ca^{2+}]_i$ and τ in rabbit by about 45 %, but more modestly in rat (i.e. $\Delta[Ca^{2+}]_i$ is not changed and τ is only increased by 20 %, in agreement with voltage clamp studies; Bers *et al.* 1990). The large effects in rabbit cells indicate that Ca^{2+} extrusion via Na^+–Ca^{2+} exchange must be occurring during the rise of $[Ca^{2+}]_i$ at a rate sufficient to limit the rise of free $[Ca^{2+}]_i$.

SR Ca^{2+}-ATPase in relaxation

The decline of $[Ca^{2+}]_i$ during a twitch with Na^+–Ca^{2+} exchange blocked was 2- to 3-fold faster in rat than in rabbit myocytes. Since this relaxation is dominated by the SR Ca^{2+}-ATPase, we infer that the SR Ca^{2+}-ATPase is 2- to 3-fold faster in rat, which agrees with recent measurements of SR Ca^{2+} transport in permeabilized rat and rabbit myocytes (Hove-Madsen & Bers, 1993*a*). This is the exact opposite of the above conclusion about the Na^+–Ca^{2+} exchanger.

Inhibition of the SR Ca^{2+}-ATPase during a twitch slows relaxation of the control twitch and $[Ca^{2+}]_i$ decline in both rat and rabbit, but much more so in the rat (~9-fold *vs.* ~2-fold in rabbit). This is probably because the Na^+–Ca^{2+} exchange better compensates for the weaker SR Ca^{2+}-ATPase activity in the rabbit.

The faster decline of $[Ca^{2+}]_i$ during the normal twitch in rat also emphasizes that the stronger SR Ca^{2+}-ATPase more than compensates for the weaker Na^+–Ca^{2+} exchange. From the data in Tables 1 and 3 we would infer that the SR Ca^{2+}-ATPase is ~9 times faster than the Na^+–Ca^{2+} exchange in rat, but only 2–3 times faster in the rabbit myocytes. Thus the SR Ca^{2+}-ATPase is dominant in both species, but more markedly so in rat.

Quantitative interpretations

During control twitches all of the Ca^{2+} transport systems discussed are active simultaneously, so the values of τ for $[Ca^{2+}]_i$ decline of individual systems above do not necessarily indicate the quantitative contributions during a normal twitch. It would be useful to know what fractions of the total Ca^{2+} in the myoplasm (free + bound) are transported by the SR Ca^{2+}-ATPase, the Na^+–Ca^{2+} exchange and the slow systems (sarcolemmal Ca^{2+}-ATPase and mitochondrial Ca^{2+} uniporter). We can estimate this by first determining the $[Ca^{2+}]_i$ dependence of each transport system and then allowing them to work together during a normal twitch.

First we assume that free $[Ca^{2+}]_i$ is in rapid equilibrium with passive buffers in the cell and use Ca^{2+} buffering characteristics measured directly in rabbit ventricular myocytes (Hove-Madsen & Bers, 1993*b*). These measurements also appear to be satisfactory for rat myocytes (Hove-Madsen & Bers, 1993*a*). Thus the total myoplasmic $[Ca^{2+}]$,

$$[Ca^{2+}]_t = [Ca^{2+}]_i + \frac{B_{max1}}{1 + (K_1/[Ca^{2+}]_i)} + \frac{B_{max2}}{1 + (K_2/[Ca^{2+}]_i)} + \frac{[indo]_i}{1 + (K_{in}/[Ca^{2+}]_i)}, \quad (1)$$

where B_{max1}, B_{max2}, K_1 and K_2 are empirical constants for cellular calcium buffering (from Hove-Madsen & Bers, 1993*b*). The last term reflects Ca^{2+} binding to intracellular indo-1 (assuming $[indo]_i = 50\ \mu M$ and $K_{in} = 250$ nM). Then we can convert the free $[Ca^{2+}]_i$ to $[Ca^{2+}]_t$ and differentiate it to obtain a rate of Ca^{2+} transport. During relaxation we assume that the rate of Ca^{2+} removal from the cytosol can be given by

$$d[Ca^{2+}]_t/dt = J_{SR} + J_{NaCaX} + J_{slow} - L, \quad (2)$$

where the J terms refer to flux through the SR Ca^{2+}-ATPase, Na^+–Ca^{2+} exchange and slow transporters respectively and L is a constant Ca^{2+} leak into the cytoplasm (assumed to be small compared to other fluxes during $[Ca^{2+}]_i$ decline). J_{SR}, J_{NaCaX} and J_{slow} can be described as simple quasi-empirical $[Ca^{2+}]$ dependent expressions of the form

$$J_x = \frac{V_{max}}{1 + (K_m/[Ca^{2+}]_i)^n}. \quad (3)$$

We first fitted J_{slow} by using the decline of $[Ca^{2+}]_i$ during a twitch in 0 Na^+, 0 Ca^{2+} + TG (or a caffeine-induced contracture in 0 Na^+, 0 Ca^{2+}) and setting J_{SR} and J_{NaCaX} in eqn (2) to zero. Thus we determine the constants V_{max} (maximum velocity), K_m (Michaelis–Menten constant) and n (Hill coefficient) for J_{slow}. Then we hold these values for

J_{slow} constant and similarly determine either J_{SR} or J_{NaCaX} (using a twitch $[\text{Ca}^{2+}]_i$ transient in either 0 Na^+ or TG respectively) leaving out the appropriate term in eqn (2). Finally we can simulate the action of all systems working simultaneously during a control $[\text{Ca}^{2+}]_i$ transient and integrate the individual contributions to the total flux in eqn (2).

The results of these calculations are shown in Fig. 8*C* and *D*. Figure 8*A* and *B* show calculated free $[\text{Ca}^{2+}]$ declines based on the mean curve fit parameters in Tables 2 and 3 for the various conditions. It can be seen that during a normal twitch the proportions of Ca^{2+} transported by the SR, Na^+–Ca^{2+} exchange and slow systems are 70, 28 and 2% respectively in rabbit and 92, 7 and 1% in rat myocytes. While these are probably useful quantitative estimates, they could be refined by better indo-1 calibrations and better knowledge of the characteristics of the individual transport systems. For example, eqn (3) may be appropriate for the SR Ca^{2+}-ATPase and the parameters we get (V_{max}, K_{m} and n) agree with other methods (see legend). However, this function may not be appropriate for the Na^+–Ca^{2+} exchanger (especially because of membrane potential dependence) and the results should only be considered an empirical fit to our data. Nevertheless, the estimate of 28% Ca^{2+} flux by Na^+–Ca^{2+} exchange in rabbit (above) is in agreement with

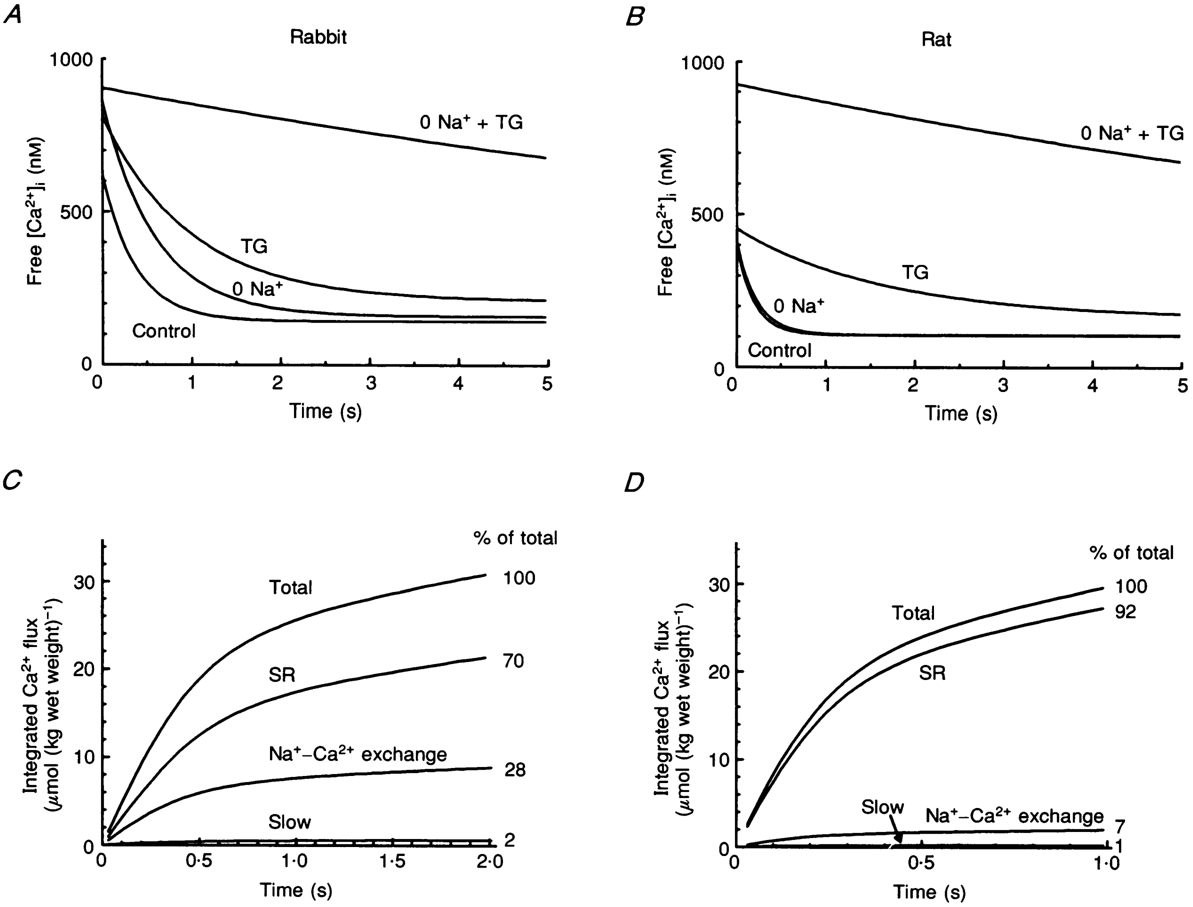

Figure 8. Decline of $[\text{Ca}^{2+}]_i$ and integrated Ca^{2+} flux during a twitch in rabbit and rat myocytes

A and *B*, decline of $[\text{Ca}^{2+}]_i$. Free $[\text{Ca}^{2+}]_i$ was calculated from mean values of τ, $\Delta[\text{Ca}^{2+}]_i$ and resting $[\text{Ca}^{2+}]_i$ in Tables 2 and 3 for the twitch conditions indicated (e.g. control). Free $[\text{Ca}^{2+}]_i$ was then converted to $[\text{Ca}^{2+}]_t$ using eqn (1) with $B_{\text{max1}} = 86$ μmol (kg wet weight)$^{-1}$, $B_{\text{max2}} = 281$ μmol (kg wet weight)$^{-1}$, $K_1 = 0{\cdot}42$ μM, $K_2 = 79$ μM (from Hove-Madsen & Bers, 1993*b*), [indo-1] = 50 μM and $K_{\text{in}} = 250$ nM. After differentiation ($\text{d}[\text{Ca}^{2+}]_t/\text{d}t$) the $[\text{Ca}^{2+}]_i$ dependence of each transport system in eqn (2) was sequentially fitted to eqn (3) as described in the text. Values obtained for J_{SR}, J_{NaCaX} and J_{slow} respectively were: $V_{\text{max}} = 32{\cdot}6$, 18·4 and 1·55 μmol (kg wet weight)$^{-1}$ for rabbit and 82·9, 10·8 and 1·6 μmol (kg wet weight)$^{-1}$ for rat; $K_{\text{m}} = 264$, 316 and 362 nM in rabbit and 184, 257 and 268 nM in rat; $n = 3{\cdot}7$, 3·7, 3·2 in rabbit and 3·9, 3·4 an 3·5 in rat. Then the Ca^{2+} flux through each system was calculated for the $[\text{Ca}^{2+}]_i$ transient decline during a normal twitch (*C* and *D*). To convert values in units of micromoles per kilogram wet weight to units of micromoles per litre of non-mitochondrial space, the B_{max} and V_{max} values should be multiplied by 2·5 (Fabiato, 1983).

previous estimates (Bers & Bridge, 1989; Hryshko *et al.* 1989). We can also extend this analysis to estimate that ~93 % of the Ca^{2+} decline during a caffeine contracture or TG twitch is removed via Na^{+}–Ca^{2+} exchange (with the rest via the slow systems).

The 7 % value for the Na^{+}–Ca^{2+} exchange contribution to [Ca^{2+}]$_i$ decline during a twitch in rat is smaller than previous indirect estimates based on time constants of relaxation or [Ca^{2+}]$_i$ decline (Bers *et al.* 1990; Crespo *et al.* 1990), but agrees with the value of 8·7 % estimated by Negretti *et al.* (1993). Results from Eisner and co-workers differ from ours in that [Ca^{2+}]$_i$ decline during caffeine contractures is a bit slower ($\tau = 1{\cdot}75$–3.0 s *vs.* our value of ~1·6 s), but is faster when the Na^{+}–Ca^{2+} exchange is also inhibited ($\tau \approx 6$ s *vs.* our value of 12 s; O'Neill *et al.* 1991; Negretti *et al.* 1993; Varro, Negretti, Hester & Eisner, 1993). It may be that mitochondrial Ca^{2+} uptake or the sarcolemmal Ca^{2+} pump is faster in their rat myocytes and possibly that the Na^{+}–Ca^{2+} exchange is slightly slower. That would be consistent with their estimate that 67 % of the Ca^{2+} flux during relaxation of a caffeine contracture goes via Na^{+}–Ca^{2+} exchange with the rest via the slow systems (where our estimate for rat myocytes is 87 %). Whether these differences are due to strain, age of rats or methodology is not clear.

It is interesting to note that the total flux of Ca^{2+} is similar in the rat and rabbit cells, while ~4 times as much Ca^{2+} is extruded by Na^{+}–Ca^{2+} exchange in rabbit myocytes. In the steady state, where Ca^{2+} influx and efflux must be matched on a beat-to-beat basis, this would require that 4 times as much Ca^{2+} influx occurs during the twitch in rabbit myocytes. While peak Ca^{2+} current does not appear to be very different in rat and rabbit cells (Bean & Ríos, 1989), the Ca^{2+} current in rat also appears to inactivate more rapidly and recovers from inactivation more slowly compared with guinea-pig myocytes (Josephson, Sanchez-Capula & Brown, 1984). These features, together with the short action potential (which contributes to early deactivation of Ca^{2+} current), may indicate that the integrated Ca^{2+} influx during a twitch in the rat is much smaller than in rabbit or guinea-pig (the latter two being functionally similar; Bers, 1991). Since Ca^{2+}-induced Ca^{2+}-release in rat is more sensitive to Ca^{2+} (Fabiato, 1982), smaller Ca^{2+} influx during the action potential may be required to activate SR Ca^{2+} release. This conclusion is also consistent with the observation that Ca^{2+} influx during the action potential (with the SR inhibited) can support large contractions in rabbit and guinea-pig, but not in rat (Sutko & Willerson, 1980; Bers, 1985; Kirby *et al.* 1992; Bassani *et al.* 1993*a*; Lewartowski & Wolska, 1993).

In conclusion we have evaluated [Ca^{2+}]$_i$ transients during twitches where either Na^{+}–Ca^{2+} exchange or the SR Ca^{2+}-ATPase were inhibited separately or simultaneously. During the decline of the [Ca^{2+}]$_i$ transient, it appears that the Na^{+}–Ca^{2+} exchange is about 2- to 3-fold faster in rabbit than in rat, whereas the SR Ca^{2+}-ATPase is 2- to 3-fold faster in the rat. While the SR Ca^{2+}-ATPase is more powerful than the Na^{+}–Ca^{2+} exchange in both cell types the dominance is much more marked in rat (i.e. ~13-fold, *vs.* 2·5-fold in rabbit).

REFERENCES

Bassani, J. W. M., Bassani, R. A. & Bers, D. M. (1993*a*). Twitch-dependent SR Ca accumulation and release in rabbit ventricular myocytes. *American Journal of Physiology* **265**, C533–540.

Bassani, J. W. M., Bassani, R. A. & Bers, D. M. (1993*b*). Ca^{2+} cycling between sarcoplasmic reticulum and mitochondria in rabbit cardiac myocytes. *Journal of Physiology* **460**, 603–621.

Bassani, R. A., Bassani, J. W. M. & Bers, D. M. (1992*a*). Mitochondrial and sarcolemmal Ca^{2+} transport reduce [Ca^{2+}]$_i$ during caffeine contractures in rabbit cardiac myocytes. *Journal of Physiology* **453**, 591–608.

Bassani, R. A., Bassani, J. W. M. & Bers, D. M. (1992*b*). The role of sarcolemmal Na–Ca exchange in extrusion of Ca from cardiac myocytes during rest: species-dependent differences. *FASEB Journal* **6**, A1487.

Bassani, R. A. & Bers, D. M. (1993). Interconversion of post-rest contractile behavior of rabbit and rat cardiac myocytes by manipulations affecting Na–Ca exchange (NaCaX). *Proceedings of the International Union of Physiological Sciences* **38**, 193.

Bean, B. P. & Ríos, E. (1989). Nonlinear charge movements in mammalian cardiac ventricular cells. *Journal of General Physiology* **94**, 65–93.

Bers, D. M. (1985). Ca influx and sarcoplasmic reticulum Ca release in cardiac muscle activation during postrest recovery. *American Journal of Physiology* **248**, H366–381.

Bers, D. M. (1991). *Excitation–Contraction Coupling and Cardiac Contractile Force*, p. 258. Kluwer Academic Press, Dordrecht, The Netherlands.

Bers, D. M., Bassani, R. A., Bassani, J. W. M., Baudet, S. & Hryshko, L. V. (1993). Paradoxical twitch potentiation after rest in cardiac muscle: increased fractional release of SR calcium. *Journal of Molecular and Cellular Cardiology* **25**, 1047–1057.

Bers, D. M. & Bridge, J. H. B. (1989). Relaxation of rabbit ventricular muscle by Na–Ca exchange and sarcoplasmic reticulum Ca-pump: ryanodine and voltage sensitivity. *Circulation Research* **65**, 334–342.

Bers, D. M., Bridge, J. H. B. & Spitzer, K. W. (1989). Intracellular Ca^{2+} transients during rapid cooling contractures in guinea-pig ventricular myocytes. *Journal of Physiology* **417**, 537–553.

Bers, D. M., Christensen, D. M. & Nguyen, T. X. (1988). Can Ca entry via Na–Ca exchange directly activate cardiac muscle contraction? *Journal of Molecular and Cellular Cardiology* **20**, 405–414.

Bers, D. M., Lederer, W. J. & Berlin, J. R. (1990). Intracellular Ca transients in rat cardiac myocytes: role of Na–Ca exchange in excitation–contraction coupling. *American Journal of Physiology* **258**, C944–954.

Beuckelmann, D. J. & Wier, W. G. (1988). Mechanism of release of calcium from sarcoplasmic reticulum of guinea-pig cardiac cells. *Journal of Physiology* **405**, 233–255.

Bridge, J. H. B., Spitzer, K. W. & Ershler, P. R. (1988). Relaxation of isolated ventricular cardiomyocytes by a voltage-sensitive process. *Science* **241**, 823–825.

CAPOGROSSI, M. C., KORT, A. A., SPURGEON, H. A. & LAKATTA, E. G. (1986). Single adult rabbit and rat cardiac myocytes retain the Ca^{2+}- and species-dependent systolic and diastolic contractile properties of intact muscle. *Journal of General Physiology* **88**, 589–613.

CRESPO, L. M., GRANTHAM, C. J. & CANNELL, M. B. (1990). Kinetics, stoichiometry and role of the Na^+–Ca^{2+} exchange mechanism in isolated cardiac myocytes. *Nature* **345**, 618–621.

FABIATO, A. (1982). Calcium release in skinned cardiac cells: variations with species, tissues and development. *Federation Proceedings* **41**, 2238–2244.

FABIATO, A. (1983). Calcium-induced release of calcium from the cardiac sarcoplasmic reticulum. *American Journal of Physiology* **245**, C1–14.

FABIATO, A. (1985). Time and calcium independence of activation and inactivation of calcium-induced release of calcium from the sarcoplasmic reticulum of a skinned canine cardiac Purkinje cell. *Journal of General Physiology* **85**, 247–290.

GRYNKIEWICZ, G., POENIE, M. & TSIEN, R. Y. (1985). A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *Journal of Biological Chemistry* **250**, 3440–3450.

HORACKOVA, M. (1989). Possible role of Na^+–Ca^{2+} exchange in the regulation of contractility in isolated adult ventricular myocytes from rat and guinea-pig. *Canadian Journal of Physiology and Pharmacology* **67**, 1525–1533.

HOVE-MADSEN, L. & BERS, D. M. (1993*a*). Sarcoplasmic reticulum Ca uptake and thapsigargin sensitivity in permeabilized rabbit and rat ventricular myocytes. *Circulation Research* **73**, 820–828.

HOVE-MADSEN, L. & BERS, D. M. (1993*b*). Passive Ca buffering and SR Ca uptake in permeabilized rabbit ventricular myocytes. *American Journal of Physiology* **264**, C677–686.

HRYSHKO, L. V., STIFFEL, V. M. & BERS, D. M. (1989). Rapid cooling contractures as an index of SR Ca content in rabbit ventricular myocytes. *American Journal of Physiology* **257**, H1369–1377.

JOSEPHSON, I. R., SANCHEZ-CAPULA, J. & BROWN, A. M. (1984). A comparison of calcium currents in rat and guinea-pig single ventricular cells. *Circulation Research* **54**, 144–156.

KIRBY, M. S., SAGARA, Y., GAA, S., INESI, G., LEDERER, W. J. & ROGERS, T. B. (1992). Thapsigargin inhibits contraction and Ca transient in cardiac cells by specific inhibition of the sarcoplasmic reticulum Ca pump. *Journal of Biological Chemistry* **267**, 12545–12551.

LEBLANC, N. & HUME, J. R. (1990). Sodium current-induced release of calcium from cardiac sarcoplasmic reticulum. *Science* **248**, 372–375.

LEWARTOWSKI, B., HANSFORD, R. G., LANGER, G. A. & LAKATTA, E. G. (1990). Contraction and sarcoplasmic reticulum Ca^{2+} content in single myocytes of guinea-pig heart: effect of ryanodine. *American Journal of Physiology* **259**, H1222–1229.

LEWARTOWSKI, B. & WOLSKA, B. M. (1993). The effect of thapsigargin on sarcoplasmic reticulum Ca^{2+} content and contractions in single myocytes of guinea-pig heart. *Journal of Molecular and Cellular Cardiology* **25**, 23–29.

MITCHELL, M. R., POWELL, T., TERRAR, D. A. & TWIST, V. W. (1987). Electrical activity and contraction in cells isolated from rat and guinea-pig ventricular muscle: a comparative study. *Journal of Physiology* **391**, 527–544.

NÄBAUER, M., CALLEWAERT, G., CLEEMAN, L. & MORAD, M. (1989). Regulation of calcium release is gated by calcium current, not gating charge, in cardiac myocytes. *Science* **244**, 800–803.

NEGRETTI, N., O'NEILL, S. C. & EISNER, D. A. (1993). The relative contributions of different intracellular and sarcolemmal systems to relaxation in rat ventricular myocytes. *Cardiovascular Research* **27**, 1826–1830.

O'NEILL, S. C., VALDEOLMILLOS, M., LAMONT, C., DONOSO, P. & EISNER, D. A. (1991). The contribution of Na–Ca exchange to relaxation in mammalian cardiac muscle. *Annals of the New York Academy of Sciences* **639**, 444–452.

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

SHAM, J. S. K., HATEM, S. N. & MORAD, M. (1993). Species difference in the density of Na^+–Ca^{2+} exchange current in cardiac myocytes. *Biophysical Journal* **64**, A396.

SHATTOCK, M. J. & BERS, D. M. (1987). Inotropic response to hypothermia and the temperature-dependence of ryanodine action in isolated rabbit and rat ventricular muscle: implications for excitation–contraction coupling. *Circulation Research* **61**, 761–771.

SHATTOCK, M. J. & BERS, D. M. (1989). Rat vs. rabbit ventricle: Ca flux and intracellular Na assessed by ion-selective microelectrodes. *American Journal of Physiology* **256**, C813–822.

SUTKO, J. L. & WILLERSON, J. T. (1980). Ryanodine alteration of the contractile state of rat ventricular myocardium. Comparison with dog, cat and rabbit ventricular tissues. *Circulation Research* **46**, 333–343.

VARRO, 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.

VETTER, R., KEMSIES, C. & SCHULZE, W. (1987). Sarcolemmal Na^+–Ca^{2+} exchange and sarcoplasmic reticulum Ca^{2+} uptake in several cardiac preparations. *Biomedica Biochimica Acta* **46**, S375–381.

WENDT, I. R. & STEPHENSON, D. G. (1983). Effects of caffeine on Ca-activated force production in skinned cardiac and skeletal muscle fibres of the rat. *Pflügers Archiv* **398**, 210–216.

WIER, W. G. (1990). Cytoplasmic calcium in mammalian ventricle: dynamic control by cellular processes. *Annual Review of Physiology* **52**, 467–485.

WRZOSEK, A., SCHNEIDER, H., GRUENINGER, S. & CHIESI, M. (1992). Effect of thapsigargin on cardiac muscle cells. *Cell Calcium* **13**, 281–292.

Acknowledgements

We are grateful to Ms Karen Cha for preparation of myocytes. This work was supported by a grant from USPHS (HL-30077). J. W. M. B. is on leave from the Departamento de Engenharia Biomédica, Universidade Estadual de Campinas, Campinas, SP, Brazil.

Received 1 June 1993; revised 26 August 1993; accepted 15 September 1993.