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Mitochondria regulate inactivation of L-type Ca^{2+} channels in rat heart

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1. L-type Ca^{2+} channels play an important role in vital cell functions such as muscle contraction and hormone secretion. Both a voltage-dependent and a Ca^{2+}-dependent process inactivate these channels. Here we present evidence that inhibition of the mitochondrial Ca^{2+} import mechanism in rat (Sprague-Dawley) ventricular myocytes by ruthenium red (RR), by Ru360 or by carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) decreases the magnitude of electrically evoked transient elevations of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$). These agents were most effective at stimulus rates greater than 1 Hz.

2. RR and CCCP also caused a significant delay in the recovery from inactivation of L-type Ca^{2+} currents (I_{Ca}). This suggests that sequestration of cytosolic Ca^{2+}, probably near the mouth of L-type Ca^{2+} channels, into mitochondria during cardiac contractile cycles, helps to remove the Ca^{2+}-dependent inactivation of L-type Ca^{2+} channels.

3. We conclude that impairment of mitochondrial Ca^{2+} transport has no impact on either L-type Ca^{2+} currents or SR Ca^{2+} release at low stimulation frequencies (e.g. 0.1 Hz); however, it causes a depression of cytosolic Ca^{2+} transients attributable to an impaired recovery of L-type Ca^{2+} currents from inactivation at high stimulation frequencies (e.g. 3 Hz). The impairment of mitochondrial Ca^{2+} uptake and subsequent effects on Ca^{2+} transients at high frequencies at room temperature could be physiologically relevant since the normal heart rate of rat is around 5 Hz at body temperature. The role of mitochondria in clearing Ca^{2+} in the micro-domain near L-type Ca^{2+} channels could be impaired during high frequencies of heart beats such as in ventricular tachycardia, explaining, at least in part, the reduction of muscle contractility.

Activation of the cardiac L-type Ca^{2+} channels during an action potential leads to Ca^{2+}-induced Ca^{2+} release from the sarcoplasmic reticulum (SR) (Fabiato, 1983; Beuckelmann & Wier; 1988; Bers, 1991; Eisner *et al.* 1998). These channels are subsequently inactivated by elevation of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$) as well as by membrane depolarization (Chad & Eckert, 1984; Kass & Sanguinetti, 1984; Lee *et al.* 1985; Imredy & Yue, 1994). Therefore, $[Ca^{2+}]_c$ in the vicinity of the channels critically controls their activity during excitation–contraction (EC) coupling.

The major factor contributing to channel inactivation in intact heart cells is Ca^{2+} released from the SR, with a small contribution from Ca^{2+} permeating through the channel itself (Satomi *et al.* 1996; Sun *et al.* 1997). Although the source of Ca^{2+} that inactivates L-type Ca^{2+} channels is well characterized, less is known about the mechanisms that clear cytosolic Ca^{2+} near the channel. *In situ*, mitochondria are located in tight apposition with endoplasmic reticulum (ER)/SR and can preferentially sequester the Ca^{2+} released from ER/SR (Rizzuto *et al.* 1993; Hajnóczky *et al.* 1995; Sharma *et al.* 2000). Furthermore, inhibitors of mitochondrial Ca^{2+} uptake severely compromise clearance of cytosolic Ca^{2+} after an imposed Ca^{2+} elevation in neurons (Thayer & Miller, 1990; Babcock & Hille, 1998). A similar role of mitochondria during the heartbeat is less likely, since the SR Ca^{2+} pump is the major system that removes Ca^{2+} from the cytosol (Bassani *et al.* 1994). However, recent work has revealed a preferential coupling of Ca^{2+} transport in rat ventricular myocytes from SR to mitochondria, because of their structural proximity (Sharma *et al.* 2000). Therefore, mitochondria might influence the local $[Ca^{2+}]_c$ near L-type Ca^{2+} channels and thus modify their inactivation. To test this hypothesis, we monitored L-type Ca^{2+}

currents (I_{Ca}) in cardiac myocytes and compared their rates of recovery from inactivation in the presence and absence of inhibitors of mitochondrial Ca^{2+} uptake.

METHODS

Experiments upon rats were performed according to protocols approved by the Division of Laboratory Animal Medicine, University of Rochester, in compliance with state law, federal statute and NIH policy.

Cells and solutions

The method for myocyte isolation from adult rats has been described previously (Sharma *et al.* 2000). Briefly, isolated myocytes were prepared from the ventricles of adult male Sprague-Dawley rats (250–300 g, 5–6 months old). The rats were anaesthetized with sodium pentobarbital (60 mg (kg body weight)$^{-1}$, I.P.). Rats were checked for deep anaesthesia by testing for tail and ear pinch reflexes. Only after making sure that the rat was under deep anaesthesia, the heart, along with a 5 mm section of the aortic arch, was quickly excised. The heart was mounted on a plastic cannula inserted into the aorta and perfused with Ca^{2+}-free Joklik's tissue culture medium (Gibco, Grand Island, NY, USA) for 5 min to cleanse the heart of blood. The perfusion solution was then changed to Joklik's medium containing 50 μM $CaCl_2$, 0.5 mg ml^{-1} collagenase (Worthington, Lakewood, NJ, USA, type II) and 0.1 % BSA (Sigma Chemical Co., St Louis, MO, USA). This enzyme solution was recirculated through the heart for approximately 30 min. The ventricles collected in separate flasks were shaken vigorously and filtered through 200 μm nylon mesh to obtain dissociated single cells. The isolated myocytes were kept in standard solution that contained (mM): NaCl 140, KCl 5, $CaCl_2$ 2, $MgCl_2$ 2, Hepes 10 and glucose 11, pH 7.4 at 37 °C with NaOH.

Measurement of cytosolic and mitochondrial [Ca^{2+}]

The method for recording of $[Ca^{2+}]_c$ from single cardiac myocytes has been previously published (Sharma *et al.* 1996). Briefly, isolated myocytes were loaded with 3 μM fura-2 AM for 15 min at room temperature in standard Tyrode solution. After loading, the cells were incubated in dye-free solution for over 45 min to allow the conversion of dye to its Ca^{2+}-sensitive, free acid form. The coverslip with dye-loaded cells was mounted in a tissue chamber on the stage of a Nikon Diaphot inverted microscope equipped for epifluorescence (Deltascan 1, Photon Technology International, Princeton, NJ, USA). To evoke Ca^{2+} transients, myocytes were electrically stimulated at various frequencies with an S8 Grass stimulator. The cell was sequentially excited at 340 and 380 nm wavelength light using two excitation monochromators at a switching frequency of 100 Hz controlled by an optical chopper. The emission fluorescence was collected at 510 nm. The results are then presented as the ratio (R) of fluorescence excited at 340 nm (F_{340}) to that excited at 380 nm (F_{380}). The amplitude of each individual Ca^{2+} transient is defined as the difference between the systolic (peak) fluorescence ratio values and diastolic (baseline) fluorescence ratio values. To minimize the variation, amplitudes of six consecutive transients recorded at the steady state were averaged to produce the representative Ca^{2+} transient amplitude at a particular frequency of stimulation.

The details of mitochondrial calcium concentration ($[Ca^{2+}]_m$) and $[Ca^{2+}]_c$ measurements in chemically skinned myocytes with rhod-2 and fura-2, respectively, have been described in detail before (Sharma *et al.* 2000). Rhod-2 has been used to measure changes of $[Ca^{2+}]_m$ in living cells (Hajnóczky *et al.* 1995; Jou *et al.* 1996). One of the requirements for using this technique to measure $[Ca^{2+}]_m$ is that the recorded fluorescence signals for Ca^{2+} must originate solely from mitochondria. To achieve this, intact myocytes were first loaded with the dye by incubating the cells in standard solution containing rhod-2 AM (2 μM, Molecular Probes, Eugene, OR, USA) for 40–50 min at room temperature. Since rhod-2 AM consists of a cationic rhodamine molecule, it accumulates preferentially inside the mitochondria due to their negative membrane potential. After loading, the cells were kept in rhod-2 AM-free standard solution for at least 1 h to allow conversion of the dye to its Ca^{2+}-sensitive, free acid form. A droplet of the suspension of cells was then transferred to the laminin-coated Lab-Tek perfusion chamber (VWR, Rochester, NY, USA). To remove residual rhod-2 in the cytosol, the plasma membrane was skinned by exposing the myocytes for 20 s with a solution containing (mM): KCl 120, NaCl 10, glucose 10, $MgCl_2$ 2, Na_2ATP 5, Na_2CrP 15, succinate 5, EGTA 0.1, Hepes 10 and saponin 0.2 mg ml^{-1}, pH 7.20 (Neary *et al.* 1996). The myocytes were then perfused with the above buffer containing 100 nM free Ca^{2+} but without saponin to wash away rhod-2 that leaked out from cytosol after the plasma membrane was permeabilized. The free Ca^{2+} concentrations were calculated according to a computer program developed by Fabiato & Fabiato (1979). The cells were then exposed to the excitation light of 555 nm wavelength and the emission fluorescence was collected at 590 nm (Sharma *et al.* 2000). Since rhod-2 is not a ratiometric dye, its fluorescence intensity was not calibrated to obtain absolute values of $[Ca^{2+}]_m$. All fluorescence signals (F) are expressed relative to the control value (F_0).

For measurements of $[Ca^{2+}]_c$ in chemically skinned myocytes, 10 μM pentapotassium salt of fura-2 was added to the incubating solution. Similar to the measurements of $[Ca^{2+}]_c$ in intact myocytes, the results were presented as ratio values ($R = F_{340}/F_{380}$). In some cases, *in situ* calibration was done to convert the ratio values into absolute values of $[Ca^{2+}]_c$. Permeabilized myocytes were perfused with calibration solution containing (mM): KCl 140, NaCl 10, K_2EGTA 1, $MgCl_2$ 1, Hepes 10 and 10 μM fura-2 pentapotassium salt, adjusted to pH 7.2. Two calibrating solutions, containing 0 and 0.1 mM Ca^{2+}, were used to determine the R_{min} and R_{max}, respectively. $[Ca^{2+}]_c$ values were calculated from the 340 to 380 nm ratios using the following equation:

$$[Ca^{2+}]_c = K_d\left(\frac{R - R_{min}}{R_{max} - R}\right)\left(\frac{S_{f2}}{S_{b2}}\right),$$

where R is the measured ratio between F_{340} and F_{380} and R_{min} and R_{max} are the ratios obtained in solutions containing 0 Ca^{2+} and 0.1 mM Ca^{2+}, respectively. S_{f2} is the 380 nm excitation signal in 0 Ca^{2+}, and S_{b2} is the 380 nm excitation signal at 0.1 mM Ca^{2+} in calibrating buffer. K_d is the dissociation constant for fura-2–Ca^{2+} and taken to be 224 nM (Grynkiewicz *et al.* 1985).

Electrophysiological techniques

Inward Ca^{2+} currents were recorded from rat myocytes. To record membrane currents, the patch-clamp technique was used in the whole-cell configuration (Hamill *et al.* 1981). Pipettes were double-pulled from hard glass (KIMAX-51, Kimble Glass, Toledo, OH, USA) using a horizontal Brown-Fleming puller. Seventy to ninety per cent of the series resistance was compensated.

Membrane currents in response to voltage-step depolarizations applied from the holding potential were measured with an EPC-7 amplifier (List Electronics, Darmstadt, Germany) and sampled by a microcomputer. Analog signals were digitized with a Labmaster interface (Axon Instruments, Foster City, CA, USA) at a 12 bit resolution. Currents were amplified and filtered with an active four-pole, low-pass Bessel filter with a corner frequency of no more than half the sampling frequency. To measure activation of Ca^{2+} currents, command pulses of 50–100 ms duration and variable amplitude were delivered. Linear currents were subtracted with the $P/-6$ procedure. Data were analysed by a combination of pCLAMP 6.0 (Axon Instruments) and in-house software. The fitting of numerical

formulae to the experimental data employed a non-linear least squares algorithm. The pipette solution contained (mM): caesium aspartate 100, CsCl 20, TEACl 20, Mg-ATP 5, $MgCl_2$ 1, EGTA 0.05, Hepes 5, pH 7.2. Previous work has shown that mitochondrial Ca^{2+} transport capabilities are not impaired in the presence of high Cs^+ solutions (Herrington *et al.* 1996; Zhou *et el.* 1998; see also Results). The standard bath solution contained (mM): NaCl 140, KCl 5, $CaCl_2$ 1, $MgCl_2$ 2, glucose 10 and Hepes 10, pH 7.5.

All experiments were carried out at room temperature (23 °C).

RESULTS

First, we established the experimental conditions that allow a selective blockade of mitochondrial Ca^{2+} uptake in rat ventricular myocytes. For this we used ruthenium red (RR), a blocker of the mitochondrial uniporter in several cell types including heart cells (McCormack & England, 1983; Hansford, 1987; Trollinger *et al.* 2000). Alternatively, we used Ru360, a selective inhibitor of mitochondrial uniporter in single cardiac myocytes (Matlib *et al.* 1998), or CCCP. In CCCP experiments, oligomycin was added to remove the voltage gradient for Ca^{2+} influx through mitochondrial uniporter without ATP depletion (Nieminen *et al.* 1990; Budd & Nicholls, 1996). Figure 1*A* and *B* illustrates that RR (1–10 μM) blocks the mitochondrial Ca^{2+} uptake, confirming previous results (Sharma *et al.* 2000). In permeabilized myocytes, caffeine (10 mM) caused a rapid increase (4.10 ± 0.37-fold, $n = 14$) in peak rhod-2 fluorescence (Fig. 1*A*). RR (4 μM) significantly blocked the caffeine response. The increase in the rhod-2 fluorescence signal in response to caffeine was only 0.06 ± 0.01-fold (Fig. 1*B*, $n = 14$), consistent with results from a previous report in digitonin-permeabilized HeLa cells (Rizzuto *et al.* 1993). To test the effects of RR on Ca^{2+} release from SR, we measured $[Ca^{2+}]_c$ in skinned cardiac myocytes using the fluorescent indicator fura-2. Under control conditions (Fig. 1*C*), caffeine produced an immediate transient increase in fluorescence. A similar response was obtained with 4 μM RR (Fig. 1*D*). Peak $[Ca^{2+}]_c$ levels were 412 ± 17 nM ($n = 14$) in control conditions and 459 ± 13 nM ($n = 5$) in the presence of RR. Likewise, the resting $[Ca^{2+}]_c$ levels were 87 ± 4 nM ($n = 14$) and 92 ± 4 nM ($n = 5$), respectively. These results agree with previous work indicating that 4 μM RR has no significant effect on caffeine-induced contractures or on Ca^{2+}-induced Ca^{2+} release in heart (Zhu & Nosek, 1992; Sharma *et al.* 2000). Finally, we tested the effects of RR on the caffeine-induced $[Ca^{2+}]_c$ response in intact cells. Caffeine (20 mM) produced a robust increase in $[Ca^{2+}]_c$ (Fig. 1*E*). After that, the cell was incubated with normal solution containing RR (10 μM) for 12 min and tested again. Myocytes were pretreated with RR or Ru360 for a period of 12 min as these chemicals permeate the intact plasma membrane slowly. Matlib *et al.* (1998) have shown that it takes at least 10 min for externally applied Ru360 to achieve a maximum concentration in isolated cardiac myocytes. RR had no effect on caffeine-induced Ca^{2+} signals in the intact myocyte (Fig. 1*F*). The ratio between the peak amplitude in response to caffeine of the RR-treated cells to that of the untreated control cells averaged 1.05 ± 0.17 ($n = 7$).

After establishing that RR does not interfere with Ca^{2+} release from SR by caffeine under the present experimental conditions, we investigated its effect on Ca^{2+} transients produced by electrical stimulation. An intact cardiac myocyte was electrically stimulated at 0.1 Hz and its cytosolic Ca^{2+} transients were recorded when the steady state was achieved (Fig. 2*A*). The stimulation was paused and the cell was exposed to 10 μM RR for at least 12 min. The stimulation was then resumed and the Ca^{2+} transients were recorded again. The electrical stimulation protocol used ensured a steady state level in the amplitude of Ca^{2+} transients as we found experimentally. The stimulation was paused to allow a complete and fast exchange of the extracellular solutions. These transients in the steady state were identical to those recorded in the control solution (Fig. 2*B*). Similar

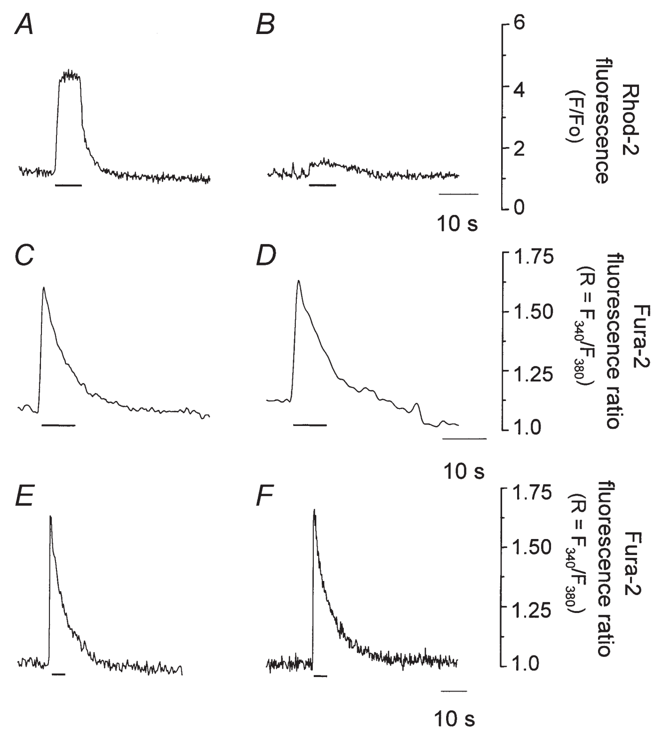

Figure 1. The effect of RR on $[Ca^{2+}]_m$ and $[Ca^{2+}]_c$ in skinned and intact cardiac myocytes during caffeine application

Mitochondrial rhod-2 signals from a skinned cell in response to caffeine (10 mM): *A*, control; *B*, after incubation in RR (4 μM). Fluorescence data are expressed relative to resting fluorescence (F/F_o). Cytosolic fura-2 ratiometric signals ($R = F_{340}/F_{380}$) from a skinned cell in response to caffeine (10 mM): *C*, control; *D*, after incubation in RR (4 μM). Cytosolic fura-2 ratiometric signals from an intact cell in response to caffeine (20 mM): *E*, control; *F*, in the presence of RR (10 μM). Horizontal bars indicate time of caffeine application.

experiments were performed in additional myocytes using higher frequencies of stimulation. Figure 2*C* illustrates Ca^{2+} transients recorded at a frequency of 3.0 Hz under control conditions. RR significantly decreased the amplitude of individual Ca^{2+} transients at this frequency of stimulation (Fig. 2*D*). Data from several cells were summarized by plotting the ratio of the mean amplitude of evoked Ca^{2+} transients recorded in the presence of RR to that recorded in its absence, as a function of the frequency of stimulation (Fig. 2*E*). The results indicate that as the frequency of stimulation increased, the ratio decreased, suggesting that blocking the mitochondrial uniporter reduces Ca^{2+} release by SR more effectively at high frequencies of stimulation.

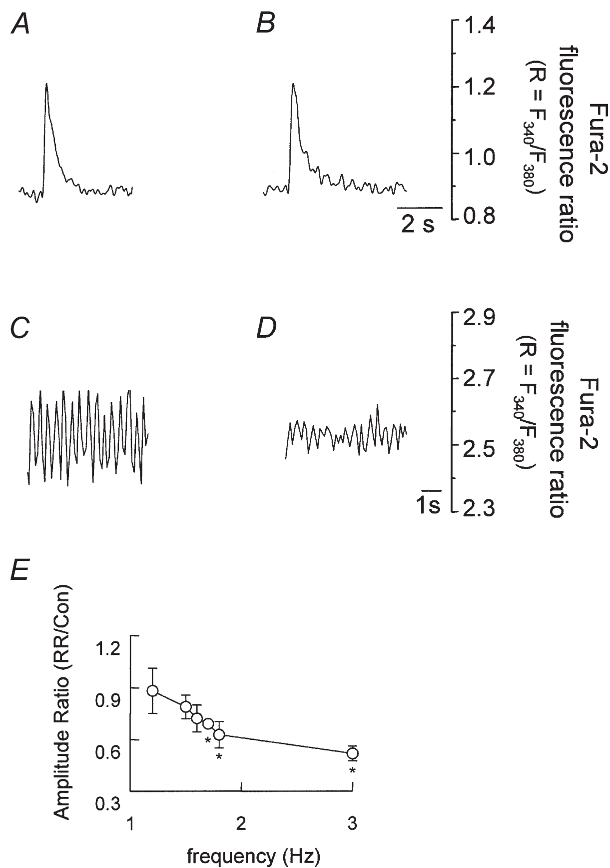

Figure 2. The effect of RR on the cytosolic Ca^{2+} transients measured with fura-2 at different frequencies of stimulation

Ca^{2+} transients from an intact cardiomyocyte electrically stimulated at 0.1 Hz: *A*, control; *B*, after incubation in RR (10 μM). Ca^{2+} transients from an intact cardiomyocyte electrically stimulated at 3.0 Hz: *C*, control; *D*, after incubation in RR (10 μM). *E*, the relationship between action of RR (ordinate) and frequency of stimulation (abscissa). The amplitude ratio is the mean amplitude of evoked $[Ca^{2+}]_c$ response observed in the presence of RR divided by that recorded in its absence. Points with error bars are the average (± S.E.M.) of 3–8 experiments. * $P < 0.05$.

Experiments with RR were further substantiated by using Ru360, a compound that inhibits mitochondrial Ca^{2+} uptake without inhibiting Ca^{2+} release from SR (Matlib *et al.*1998; Zhou *et al.* 1998). After obtaining Ca^{2+} transients at frequencies of 0.1 and 3 Hz, the stimulation was paused and the cell was exposed to 10 μM Ru360 for a period of 30 min. The stimulation was then resumed and Ca^{2+} transients were recorded again. Figure 3 shows that there was no difference in the amplitude of Ca^{2+} transients in control and Ru360-treated cells when the cell was stimulated at a frequency of 0.1 Hz (Fig. 3*A*, control; and Fig. 3*B*, with Ru360). However, the amplitude of Ca^{2+} transients was significantly reduced in a Ru360-treated cell when it was stimulated at a frequency of 3.0 Hz (Fig. 3*C*, control; and Fig. 3*D*, with Ru360). Incubation of cardiac cells with 10 μM Ru360 reduced the amplitude of Ca^{2+} transients to 68 ± 13% ($n = 5$, $P < 0.001$) compared to the amplitude of Ca^{2+} transients in untreated cells at 3 Hz of stimulation.

The effect of mitochondrial Ca^{2+} uniporter blockade upon Ca^{2+} transients was further verified by treating the cardiac cells with CCCP (5 μM) and oligomycin (5 μM), to remove the voltage gradient for Ca^{2+} influx through Ca^{2+} uniporter without ATP depletion. As shown in Fig. 4, the transients obtained at the frequency of 0.1 Hz were similar in control (Fig. 4*A*) and CCCP-treated cells (Fig. 4*B*). However, the amplitude of Ca^{2+} transients in CCCP- and oligomycin-treated cells was significantly reduced upon stimulation of the cell at a frequency of

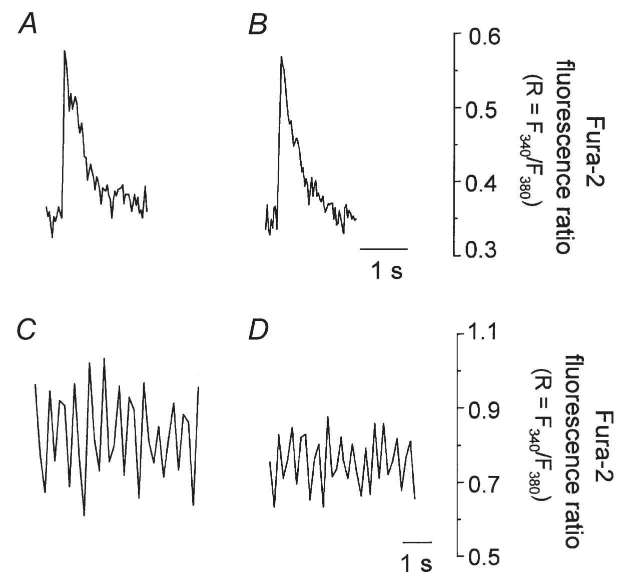

Figure 3. The effect of Ru360 on the cytosolic Ca^{2+} transients measured with fura-2 at different frequencies of stimulation

Ca^{2+} transients from an intact cardiac cell electrically stimulated at 0.1 Hz: *A*, control; *B*, after incubation in Ru360 (10 μM). Ca^{2+} transients from an intact cardiac cell electrically stimulated at 3.0 Hz: *C*, control; *D*, after incubation in Ru360 (10 μM).

3.0 Hz (Fig. 4*C*, control; and Fig. 4*D*, CCCP treated). Incubation of cardiac cells with CCCP and oligomycin reduced the amplitude of Ca^{2+} transients to $60 \pm 12\%$ ($n = 5$, $P < 0.001$), compared to the amplitude of Ca^{2+} transients in untreated cells at 3 Hz stimulation.

It is possible that blocking mitochondrial Ca^{2+} uptake decreases the clearance of cytosolic Ca^{2+} near the L-type Ca^{2+} channels, especially at high frequencies of stimulation. This would in turn increase the inactivation of the L-type Ca^{2+} channels leading to a decrease in Ca^{2+} transients. To test this hypothesis, we recorded I_{Ca} with the voltage-clamp technique (Hamill *et al.* 1981). Again, the first set of experiments was carried out in skinned rhod-2-loaded myocytes to show that the intracellular electrode solution does not affect the ability of mitochondria to accumulate Ca^{2+} and RR and CCCP do not affect I_{Ca} directly. Figure 5 shows that in rhod-2-loaded chemically skinned cardiac cells, caffeine-induced increases in the mitochondrial Ca^{2+} were similar in a cell suspended in either normal Na^+- and K^+-containing buffer (Fig. 5*A*) or patch pipette solution containing 120 mM Cs^+ and 20 mM TEACl (Fig. 5*B*, $98 \pm 9\%$, $n = 3$, $P > 0.05$). These data show that the electrode solution does not alter the ability of SR to release Ca^{2+} and mitochondria to sequester Ca^{2+}.

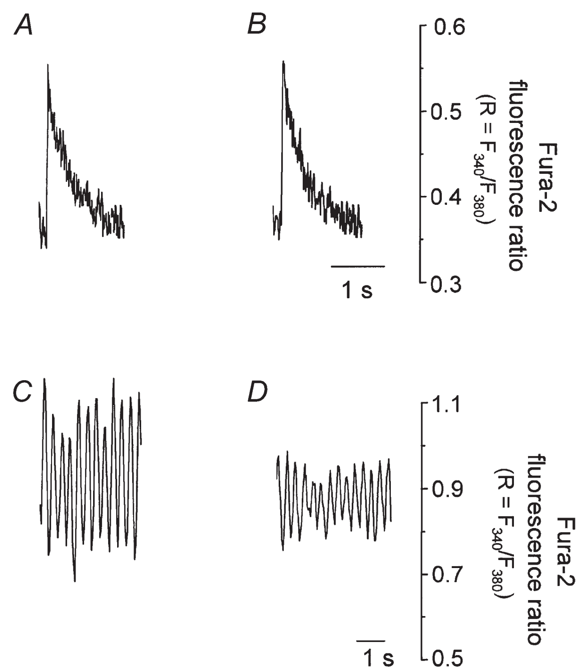

Figure 4. The effect of CCCP on the cytosolic Ca^{2+} transients measured with fura-2 at different frequencies of stimulation

Ca^{2+} transients from an intact cardiac cell electrically stimulated at 0.1 Hz: *A*, control; *B*, after incubation in CCCP (5 μM along with 5 μM oligomycin). Ca^{2+} transients from an intact cardiac cell electrically stimulated at 3.0 Hz: *C*, control; *D*, after incubation in CCCP (5 μM along with 5 μM oligomycin).

We then studied the effects of RR and CCCP on I_{Ca}. I_{Ca} was recorded during 100 ms step depolarizations at a frequency of 1 Hz during pulses from −35 mV to +95 mV in 10 mV steps from the holding potential of −40 mV. Figure 6*A* shows records of I_{Ca} obtained under control conditions during step depolarizations to −15 mV (○) and to +5 mV (●). The time course of decay of I_{Ca} was faster when the peak current was larger, as expected by a Ca^{2+}-dependent inactivation process. This was further confirmed by the experiments showing that the inactivation of Ca^{2+} channels was greatly reduced when Ba^{2+} was used as charge carrier. In four separate experiments, the ratio between the Ba^{2+} current at the end of a 45 ms depolarizing pulse to +5 mV, relative to its peak amplitude, averaged 0.99 ± 0.01 ($n = 4$), indicating very little inactivation. This is consistent with previous reports describing that entry of Ba^{2+} through calcium channels fails to trigger the release of Ca^{2+} from SR to induce Ca^{2+} channel inactivation (Nabauer *et al.* 1989) even though Ba^{2+} might have some effects on Ca^{2+} transport mechanisms in mitochondria or SR (Akerman *et al.* 1977). In the presence of RR or CCCP (plus oligomycin, 5 μM), we found that the relationship between the time course of decay of I_{Ca} and the peak amplitude of the current was similar to that in control conditions (Fig. 6*B* and *C*). The decay phase of the currents at each potential was fitted to a single exponential and the corresponding time constant was plotted as a function of the peak amplitude of I_{Ca}. Figure 6*D* (control), *E* (RR) and *F* (CCCP) summarizes data from these experiments ($n = 6$). As the current amplitude increased (*x*-axis), the time constant of decay decreased (*y*-axis) with slopes (ms (pA $pF^{-1})^{-1}$) of −1.98 (○), −1.73 (▽) and −1.63 (□), indicating no major differences in the rate of inactivation of I_{Ca} by these compounds, at all voltages tested. Since channel

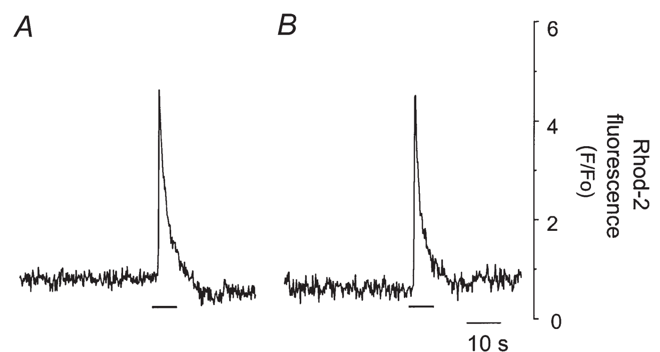

Figure 5. The effect of internal electrode solution upon caffeine-induced $[Ca^{2+}]_m$ increase

Rhod-2-loaded and chemically skinned cardiac cells were suspended in either normal Hepes buffered solution (*A*), or internal electrode solution (*B*). Horizontal bars indicate time of caffeine application (10 mM). The caffeine-induced $[Ca^{2+}]_m$ increase was similar in cardiac cells suspended in control or internal electrode solution.

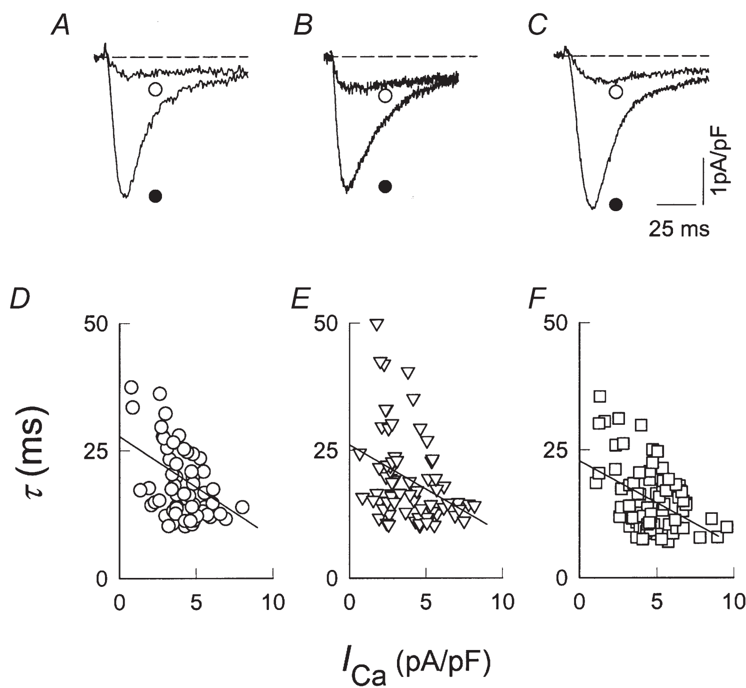

Figure 6. Blockade of mitochondrial uniporter and I_{Ca}

I_{Ca} was recorded in control conditions (*A*), in the presence of RR (10 μM) (*B*), and in the presence of CCCP (5 μM) plus oligomycin (5 μM) (*C*). Currents were activated by stepping the membrane potential from a holding potential of −40 mV to −15 mV (○) and to +5 mV (●). *D–F*, the relation between the time constant of decay and the amplitude of I_{Ca} under control conditions (○, $n = 14$), in RR- (▽, $n = 18$) and in CCCP-incubated (□, $n = 28$) cells. Straight lines have slopes (ms (pA $pF^{-1})^{-1}$) of −1.98 in *D*, −1.73 in *E* and −1.63 in *F*.

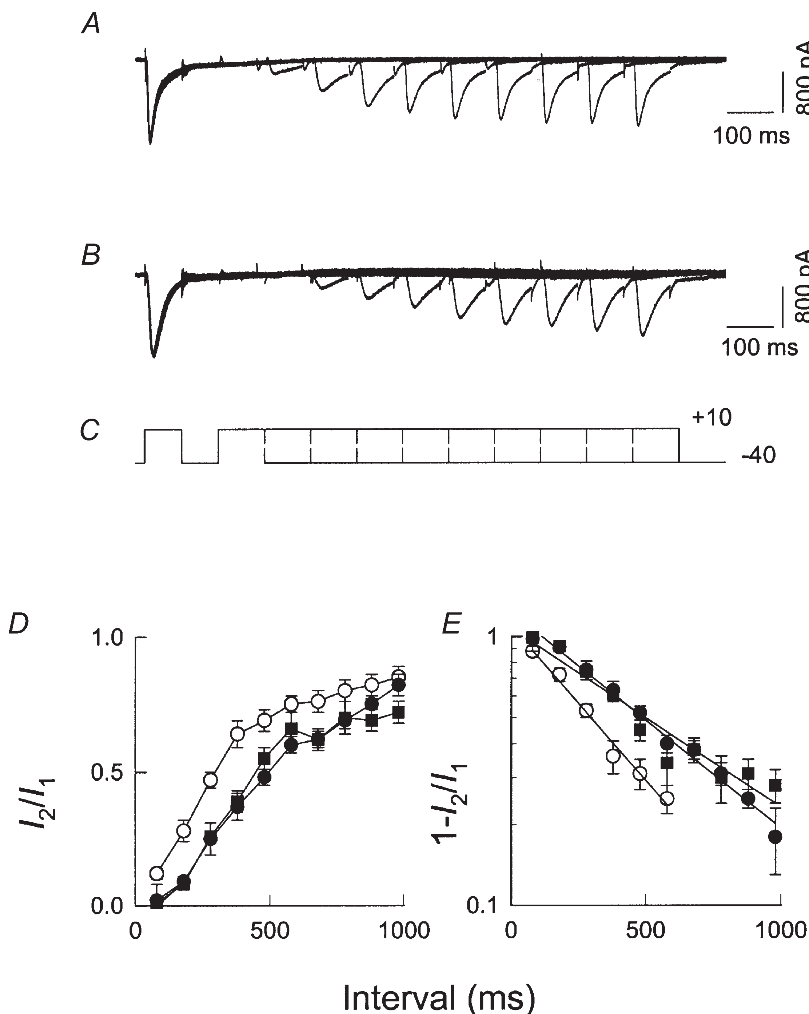

Figure 7. The effect of mitochondrial uniporter inhibition on the recovery of I_{Ca} from inactivation

I_{Ca} was produced by pairs of identical pulses delivered at intervals varying from 100 to 1000 ms. The holding potential was −40 mV and the test potential was +10 mV. Families of I_{Ca} produced by each paired pulse were superimposed to show the time-dependent recovery from inactivation. *A*, control; *B*, a separate experiment in a cell incubated in RR (10 μM); *C*, the pulse protocol. *D*, the amplitude of I_{Ca} during the second pulse (I_2) relative to its value during the first pulse (I_1), as a function of the time interval between pulses, under control conditions (○), in cells incubated with RR (10 μM) (●), and in cells incubated in (10 μM) CCCP (■). Each point is the mean (± S.E.M.) of at least four determinations. *E*, fraction of inactivated channels plotted semi-logarithmically against pulse interval from the same experiments as in *D*. The time constants for recovery from inactivation are 300 ms (○, control), 540 ms (●, RR) and 654 ms (■, CCCP).

inactivation is dependent on $[Ca^{2+}]_c$, these results suggest that Ca^{2+} release from SR is not affected under these experimental conditions. Furthermore, they also suggest that RR or CCCP does not have a direct action on the L-type Ca^{2+} channel itself. In agreement with this conclusion, no changes in the amplitude of I_{Ca} were observed when the currents were recorded at step potentials to +5 mV in cells treated with RR or CCCP and stimulated at a frequency of 0.1 Hz. Thus, peak I_{Ca} averaged (pA pF^{-1}) -4.39 ± 0.56 ($n = 7$) in control experiments, -4.94 ± 0.51 ($n = 14$) in RR-incubated cells, and -4.45 ± 0.72 ($n = 9$) in CCCP-treated cells.

We then studied the effect of RR and CCCP on the recovery from inactivation of I_{Ca}, elicited with pulse frequencies higher than 1 Hz. Figure 7*A* shows superimposed records of I_{Ca} from a control experiment. To evaluate the recovery from inactivation, currents were elicited by the double-pulse protocol shown in Fig. 7*C* in which the interval separating two similar pulses was progressively increased from 100 to 1000 ms (Brehm *et al.* 1980). The first pulse elicited a maximal I_{Ca} that decayed during the pulse, as shown previously. The amplitude of I_{Ca} during the second pulse was critically dependent on the duration of the interval elapsed since the end of the first pulse. When the interval was short, the current was small and decayed more slowly. In the presence of RR, currents showed a decreased recovery from inactivation at every given interval as shown in Fig. 7*B*, especially after short intervals. A similar effect was observed with CCCP (with oligomycin added). Figure 7*D* illustrates the recovery of I_{Ca} as a function of the interval between pulses, in control experiments (○), and in the presence of RR (●) or CCCP (■). The recovery from inactivation was delayed by approximately 100 ms after mitochondrial Ca^{2+} influx was impaired in either way. Using the same data, the fraction of inactivated channels was plotted in a semilogarithmic scale against pulse interval (Fig. 7*E*). The graph indicates that recovery from inactivation followed a single exponential with time constants of 300 ms (○, control), or 540 ms (●, RR) and 654 ms (■, CCCP). Therefore, inhibition of mitochondrial Ca^{2+} uptake lengthened the time constant by as much as 50%. The fact that only the recovery from inactivation is altered by inhibition of mitochondrial Ca^{2+} uptake while the time constants of the Ca^{2+} current itself are unaffected suggests that clearance of focal Ca^{2+} by mitochondria proceeds more slowly than the activation and inactivation of the channels during one single pulse.

DISCUSSION

Ultrastructural studies (Sharma *et al.* 2000) have shown that Ca^{2+} release units in cardiomyocytes are preferentially located in close proximity to mitochondria. The nearest distance between them is in the 37–270 nm range. At such a distance, mitochondria are exposed to a transient Ca^{2+} elevation in the tens of micromolar range during Ca^{2+} release from SR/ER (Smith & Augustine, 1988; Neher, 1998). Since Ca^{2+} influx through the uniporter is half-maximally activated when free Ca^{2+} reaches values in that range (Gunter *et al.* 1994), mitochondria are expected to take up Ca^{2+} during Ca^{2+} transients associated with action potentials. This focal Ca^{2+} uptake by mitochondria in intact cardiomyocytes is supported by recent experimental evidence showing that mitochondria are depolarized by spontaneous Ca^{2+} release from SR (Ca^{2+} sparks) (Duchen *et al.* 1998).

At full velocity, mitochondria are capable of lowering free cytosolic Ca^{2+} at a rate of 4.6 $\mu M\ s^{-1}$ in chromaffin cells, where mitochondria account for 6% of the cell volume (Babcock *et al.* 1997). These numbers are expected to be higher in rat cardiomyocytes where the volume occupied by mitochondria is 35% (Sommer & Johnson, 1979). The distance between mitochondria and the centre of T-tubules averages 145 nm and the distance between nearest SR T tubule junctions and mitochondria averages 37 nm (Sharma *et al.* 2000). This relatively short distance makes it feasible for the mitochondria to reduce $[Ca^{2+}]_c$, mostly due to Ca^{2+} release from SR, near the L-type Ca^{2+} channel, and thereby reduces Ca^{2+}-dependent inactivation. The notion that mitochondrial buffering of Ca^{2+} close to the mouth of the channel could regulate channel inactivation has also been observed in other preparations (Budd & Nicholls, 1996; Hoth *et al.* 1997). This spatial characteristic of mitochondrial Ca^{2+} signalling, although complex, could be quite critical in determining the functional diversities that exist intrinsically in different cell types (Duchen, 1999).

Recently, it has been described that the blockade of the mitochondrial Ca^{2+} uptake mechanism in chromaffin cells causes a reduction in the amplitude of Ca^{2+} currents during depolarizing steps through L and other types of Ca^{2+} channels (Hernandez-Guijo *et al.* 2001). This reduction is due to inactivation of the channels by a Ca^{2+}-dependent mechanism. The difference between these results and ours can be explained considering the fact that in chromaffin cells, mitochondria play a major role buffering the bulk cytosolic Ca^{2+} concentration. Thus, CCCP causes a distinct increase of cytosolic Ca^{2+} in non-stimulated cells (Babcock *et al.* 1997; Hernandez-Guijo *et al.* 2001). This increase in the levels of Ca^{2+} leads to inactivation of the channels (Hernandez-Guijo *et al.* 2001). This role of mitochondria holds for chromaffin cells and neurons but not for muscle cells with a highly elaborated sarcoplasmic reticulum.

Our results are consistent with the picture that at high frequencies, mitochondria play a significant role in buffering $[Ca^{2+}]_c$ near L-type channels leading to a lesser degree of channel inactivation. From this perspective, mitochondria could be extremely important in ensuring the ability of the heart to contract effectively during rate increases. When the uniporter is blocked, the Ca^{2+} buffering capacity of mitochondria is compromised and so

is EC coupling, because of the decrease in the recovery from inactivation of the L-type channel. It is important to point out that the effect of RR on Ca^{2+} transients might conceivably be explained by a frequency-dependent blocking effect of RR on calcium-induced calcium release (CICR) directly. This is, however, unlikely because if RR is blocking CICR at higher frequencies, then the local $[Ca^{2+}]_c$ should decrease near the L-type channel and this would in turn inactivate the L-type channel less. This is opposite to what we observe in our voltage clamp experiments.

The use of RR as a specific blocker for the mitochondrial Ca^{2+} uniporter in intact cells is far from ideal (Duchen, 1992). Its effectiveness depends on the cell types used, time of exposure and concentration. Fortunately, several lines of evidence have shown that the use of RR in cardiac muscle cells is less problematic. In the perfused rat heart, RR (3.2 μM for 5 min) inhibits the positive inotropic agent-induced activation of pyruvate dehydrogenase, a Ca^{2+}-dependent intramitochondrial enzyme, consistent with the idea that RR blocks the mitochondrial Ca^{2+} influx (McCormack & England, 1983). Similarly, in isolated cardiac myocytes, RR (12 μM for 13 min) prevents activation of pyruvate dehydrogenase by high KCl solution (Hansford, 1987). In addition, RR does not attenuate the KCl-induced increase in $[Ca^{2+}]_c$, consistent with the idea that RR does not have an effect on the voltage-gated Ca^{2+} channels in the plasma membrane under this condition. It has been shown that RR at a concentration of 5 μM completely inhibits rat heart myocyte contraction at a high stimulation rate of 6 Hz (Griffiths, 2000). However, this study also reported that RR at low concentrations (0.5–1 μM) inhibited mitochondrial Ca^{2+} uptake in this preparation. The author concluded that the inhibition of $[Ca^{2+}]_m$ uptake was secondary to a reduction in $[Ca^{2+}]_c$ as a result of decreased Ca^{2+} release from SR, a conclusion supported by the ability of RR to inhibit cell shortening. However, in our studies as shown in Fig. 1*C*–*F* and 2*A* and *B*, RR up to 10 μM concentration had no inhibitory effect upon the capability of SR to release Ca^{2+} in response to caffeine or electrical stimulation at 0.1 Hz. Our experimental results are similar to that reported in a recent study showing that RR (10 μM for 20 min) inhibits electrically evoked mitochondrial rhod-2 fluorescence transients (1 Hz) but not cytosolic fluo-3 in rabbit cardiac myocytes (Trollinger *et al.* 2000). Our experimental results appear to be consistent with these studies showing the specificity of RR. In addition, similar results on Ca^{2+} transients were obtained with 10 μM Ru360, a concentration that was used to inhibit mitochondrial Ca^{2+} uptake without causing side effects on the other Ca^{2+} transport mechanisms in intact cardiac myocytes (Matlib *et al.* 1998).

Although it is expected that cardiac activity would lead to an increase in mitochondrial Ca^{2+}, this does not necessarily imply the presence of Ca^{2+} oscillations inside the mitochondria on a beat-to-beat basis. It remains unclear whether intramitochondrial Ca^{2+} can follow the cytosolic oscillations of Ca^{2+} during cardiac activity, and there is evidence for and against this possibility (Hüser *et al.* 2000; Trollinger *et al.* 2000). The presence of oscillations would depend not only on the Ca^{2+} influx but also on the kinetics of the Ca^{2+} efflux mechanism in heart mitochondria.

Changes in the physical architecture between L-type channels and SR Ca^{2+} release units have been shown to play a critical role in the reduction of contractile force under pathological conditions such as hypertrophy and failing heart (Gómez *et al.* 1997). The role of mitochondria in EC coupling that we propose here depends on their apposition to this refined architecture. It is conceivable that this structural integrity might also be altered in these pathological conditions so that the ability of mitochondria to regulate Ca^{2+} inactivation is compromised contributing to severe contractile dysfunction or heart failure.

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