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Inhibition of the sarcoplasmic reticulum Ca^{2+} pump with thapsigargin to estimate the contribution of Na^{+}-Ca^{2+} exchange to ventricular myocyte relaxation

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Abstract

Relaxation in the mammalian ventricle is initiated by Ca^{2+} removal from the cytosol, which is performed by three main transport systems: sarcoplasmic reticulum Ca^{2+}-ATPase (SR-A), Na^{+}-Ca^{2+} exchanger (NCX) and the so-called slow mechanisms (sarcolemmal Ca^{2+}-ATPase and mitochondrial Ca^{2+} uptake). To estimate the relative contribution of each system to twitch relaxation, SR Ca^{2+} accumulation must be selectively inhibited, usually by the application of high caffeine concentrations. However, caffeine has been reported to often cause changes in membrane potential due to NCX-generated inward current, which compromises the reliability of its use. In the present study, we estimated integrated Ca^{2+} fluxes carried by SR-A, NCX and slow mechanisms during twitch relaxation, and compared the results when using caffeine application (Cf-NT) and an electrically evoked twitch after inhibition of SR-A with thapsigargin (TG-TW). Ca^{2+} transients were measured in 20 isolated adult rat ventricular myocytes with indo-1. For transients in which one or more transporters were inhibited, Ca^{2+} fluxes were estimated from the measured free Ca^{2+} concentration and myocardial Ca^{2+} buffering characteristics. NCX-mediated integrated Ca^{2+} flux was significantly higher with TG-TW than with Cf-NT (12 *vs* 7 μM), whereas SR-dependent flux was lower with TG-TW (77 *vs* 81 μM). The relative participations of NCX (12.5 *vs* 8% with TG-TW and Cf-NT, respectively) and SR-A (85 *vs* 89.5% with TG-TW and Cf-NT, respectively) in total relaxation-associated Ca^{2+} flux were also significantly different. We thus propose TG-TW as a reliable alternative to estimate NCX contribution to twitch relaxation in this kind of analysis.

Key words
- Myocardium
- Relaxation
- Calcium fluxes
- Sodium-calcium exchanger
- Sarcoplasmic reticulum calcium ATPase
- Caffeine

Cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) is the key factor that determines the degree of myofilament activation, and thus contraction, in striated muscle cells. In cardiac myocytes, electric activation promotes Ca^{2+} influx, which triggers Ca^{2+} release from the sarcoplasmic reticulum (SR), resulting in increased $[Ca^{2+}]_i$ and a subsequent contraction. On the other hand, the very increase in $[Ca^{2+}]_i$ enhances Ca^{2+} transport via several

pathways, which results in cytosolic Ca^{2+} removal and allows relaxation to develop (1,2). In mammalian ventricular myocytes, the transporters that contribute more significantly to relaxation are the SR Ca^{2+}-ATPase (SR-A, which pumps Ca^{2+} from the cytosol to the SR lumen) and the Na^{+}-Ca^{2+} exchanger (NCX, which, operating in the direct mode, extrudes Ca^{2+} from the cell). Other transporters, such as the sarcolemmal Ca^{2+}-ATPase and the mitochondrial Ca^{2+} uniporter, show a very small participation in twitch relaxation in this cell type (1-5).

To determine the relative contribution of the different Ca^{2+} transporters to relaxation of intact myocytes, we estimated the relaxation-associated Ca^{2+} flux mediated by each transport system during the decline phase of the cytosolic Ca^{2+} transient (3). For this purpose it is necessary to selectively and additively inhibit these transporters. For instance, during an electrically evoked twitch, all relaxation-promoting transporters are functional. To block the SR-dependent component of relaxation, it is possible to evoke Ca^{2+} transients by rapid application of caffeine to quiescent cells. Because caffeine at millimolar concentrations promotes SR Ca^{2+} channel opening (6), not only does it release Ca^{2+} from the SR, but also prevents significant Ca^{2+} accumulation in the organelle, which is equivalent to inhibition of the SR-A. In this case, relaxation would rely only on NCX and the so-called slow systems (i.e., sarcolemmal Ca^{2+}-ATPase and the mitochondrial Ca^{2+} uniporter). To further inhibit NCX, caffeine-evoked Ca^{2+} transients may be obtained in the absence of extracellular Na^{+} and Ca^{2+}, with only the slow systems being left uninhibited. From the $[Ca^{2+}]_i$ values during each type of specific transient and the time-course of the $[Ca^{2+}]_i$ decline, as well as from data on passive cell Ca^{2+} buffering, it is possible to estimate empirical kinetic parameters for each transporter. These parameters are used to estimate the transporter-mediated Ca^{2+} fluxes during relaxation and the relative contribution of each transporter to cytosolic Ca^{2+} removal. This approach has been employed to investigate the interplay of the different Ca^{2+} transport pathways during relaxation of myocytes from animals of different species (3,4) and in different developmental stages (7), in transgenic animals (8), and in experimental models of cardiovascular disease (9).

As already stated, NCX-dependent Ca^{2+} fluxes are estimated from Ca^{2+} transients evoked by caffeine. However, the use of caffeine to inhibit SR Ca^{2+} accumulation may present problems because: a) both velocity and direction of Ca^{2+} transport by NCX are dependent on membrane potential (V_m) (10). Thus, it is possible that the kinetics of Ca^{2+} efflux via NCX is different during caffeine application, when the cell is supposedly at electrical rest, and during a twitch, when a physiological action potential takes place. b) It has been shown that caffeine application may change V_m and even evoke action potentials in quiescent ventricular myocytes (11,12), an effect attributed to the depolarizing current generated by electrogenic Ca^{2+} efflux via NCX (12). This current (and thus, depolarization) is usually large (13) because caffeine appears to release the entire SR Ca^{2+} content (4). Although in preliminary experiments, we have detected V_m changes in response to caffeine in only a small number of cells, caffeine-evoked action potentials, when present, show an unusually prolonged time-course and are often accompanied by secondary peaks in the caffeine-evoked Ca^{2+} transient and contracture (Bassani RA, unpublished results). A solution for this problem might be applying caffeine to cells voltage-clamped at the diastolic V_m (11). However, this condition would still not reproduce the action potential developed during a control twitch. Moreover, voltage-clamp is an invasive method which may disturb the composition of the intracellular medium, although the perforated whole-cell patch-clamp approach may represent a less aggres-

sive alternative of membrane voltage control.

In the present report, we propose an alternative to the use of caffeine to estimate NCX-mediated fluxes, which is using Ca^{2+} transients obtained during electrically stimulated twitches after SR pump inhibition with thapsigargin (TG). This procedure was compared to caffeine application to the same set of rat ventricular myocytes.

Ventricular myocytes were enzymatically isolated according to Bassani et al. (3) from adult male Wistar rats (10-14 weeks old). Cells were used within 8 h from the time of isolation. Myocytes were plated onto a perfusion chamber (developed at the Centro de Engenharia Biomédica/UNICAMP) coated with a collagen solution and allowed to settle. The chamber was placed on the stage of a microscope equipped for epifluorescence measurement. Cells were perfused with modified Tyrode's solution (NT; see below for composition) at 23 ± 1ºC, and field-stimulated at 0.5 Hz (biphasic voltage pulses with amplitude 1.2 x threshold, 3-ms duration).

For $[Ca^{2+}]_i$ measurement, cells were incubated with the indicator indo-1 (5 μM, acetoxymethyl ester; Molecular Probes, Eugene, OR, USA) for 15 min. Experiments were started after indo-1 had been washed out for 30 min. Indo-1 was excited at 360 nm and emission was collected at 405 and 485 nm and corrected at each wavelength for the background fluorescence recorded in an empty microscopic field of the same size. The ratio of emitted fluorescence at 405 and 485 nm (R) was converted to $[Ca^{2+}]$ (14), as $[Ca^{2+}] = K_d \times ß\,[(R - R_{min})/(R_{max} - R)]$, where R_{min} and R_{max} (R in minimal and saturating $[Ca^{2+}]$, respectively) were determined experimentally (3), ß (ratio of emission at 485 nm at minimal and saturating $[Ca^{2+}]$) was determined according to Gomes et al. (15), and the *in vivo* K_d (apparent dissociation constant for indo-1) value of 0.844 μM (16) was used.

After cells were stimulated for 5 min, Ca^{2+} transients were recorded during three successive electrically evoked twitches for averaging. Electrical stimulation was then interrupted and the perfusate was rapidly switched to NT containing 10 mM caffeine. After caffeine washout, electrical stimulation was resumed for 5 min for replenishment of the SR Ca^{2+} stores, and then interrupted again. Cells were perfused with Na^+- and Ca^{2+}-free (0Na,0Ca) solution for 15-20 s, after which the perfusate was switched to the same solution containing 10 mM caffeine. After most of the $[Ca^{2+}]_i$ decline had taken place, NT was switched on.

Cells were treated with 5 μM TG (Calbiochem, San Diego, CA, USA) for 5 min to irreversibly inhibit the SR-A (17,18). Twitches were evoked at 0.1 Hz because $[Ca^{2+}]_i$ decline is very slow after TG treatment, and lower stimulation frequencies allow greater variation in $[Ca^{2+}]_i$ during a twitch, as well as complete decline of $[Ca^{2+}]_i$ to the resting level before the next stimulus (17). All cells were tested for residual SR Ca^{2+} uptake by caffeine application in 0Na,0Ca solution following electrical stimulation. If a TG-treated cell showed any $[Ca^{2+}]_i$ increase in response to caffeine, it was discarded due to incomplete inhibition of the SR-A.

NT had the following composition: 140 mM NaCl, 6 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM glucose and 5 mM HEPES, pH 7.4 at 23ºC with NaOH. In 0Na,0Ca solution, choline chloride and EGTA replaced NaCl and $CaCl_2$, respectively. TG was dissolved in DMSO, and the concentration of the solvent applied to the cells was <0.1%.

The basic procedure for estimation of Ca^{2+} fluxes was similar to that described by Bassani et al. (3). The four types of Ca^{2+} transients, i.e., control twitches, contractures in response to caffeine in Na^+-containing (Cf-NT) and Na^+-free medium (Cf-00), as well as twitches after TG treatment (TG-TW), were obtained in 20 myocytes. Cf-NT

Ca^{2+} transients showing non-monotonic Ca^{2+} decline were discarded. In each cell, diastolic $[Ca^{2+}]_i$ was considered as the average value recorded immediately before each transient was evoked. A single exponential function was adjusted to the decline phase of each Ca^{2+} transient for estimation of the amplitude ($\Delta[Ca^{2+}]_i$, i.e., peak minus diastolic $[Ca^{2+}]_i$) and the rate constant of $[Ca^{2+}]_i$ decline (K) of the transient. To avoid interference of the signal noise in further calculations, the same function and the estimated parameters were used to generate a "synthetic" $[Ca^{2+}]_i$ decline phase of the transient (i.e., the same exponential function that best fitted the $[Ca^{2+}]_i$ decay phase of the respective transient).

The total flux of Ca^{2+} removal from the cytosol during relaxation (J_{tot}) was assumed to be:

$$J_{tot} = J_{SR} + J_{NCX} + J_{slow} - J_L$$

where J_{SR}, J_{NCX} and J_{slow} are the Ca^{2+} fluxes during relaxation carried by SR-A, NCX and the slow transporters, respectively. J_L represents the "leakage" Ca^{2+} flux from the SR and the extracellular medium to the cytosol, and was neglected in the present calculations for the sake of simplicity (3).

To estimate J_{tot} during relaxation, it is necessary to convert the free $[Ca^{2+}]$ signal (i.e., $[Ca^{2+}]_i$) to total $[Ca^{2+}]$ ($[Ca^{2+}]_T$, i.e., the sum of the free and bound $[Ca^{2+}]$) as follows:

$$[Ca^{2+}]_T = [Ca^{2+}]_i + \{B_{max\text{-}en} \times [Ca^{2+}]_i/(K_{d\text{-}en} + [Ca^{2+}]_i)\} + \{B_{max\text{-}in} \times [Ca^{2+}]_i/(K_{d\text{-}in} + [Ca^{2+}]_i)\}$$

where $B_{max\text{-}en}$ is the maximal concentration of high-affinity, passive Ca^{2+} binding sites and $K_{d\text{-}en}$ is the apparent dissociation constant of Ca^{2+} at these sites determined in permeabilized adult rat ventricular myocytes (300 and 0.53 μM, respectively; Ref. 19); $B_{max\text{-}in}$ and $K_{d\text{-}in}$ are the parameters related to indo-1 (considered as 50 and 0.844 μM, respectively).

The relationship between the time derivative of $[Ca^{2+}]_T$ over the decline of the transient (J) and the respective $[Ca^{2+}]_i$ values was used to estimate the kinetic parameters of a given transporter x, as follows:

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

where V_{max} is the maximal transport velocity, K_m is the $[Ca^{2+}]_i$ at which velocity is 50% of V_{max}, and n is the Hill coefficient.

Thus, if this relationship is applied to the $[Ca^{2+}]_i$ decline during Cf-00 (in which both SR Ca^{2+} accumulation and NCX are inhibited, thus $J_{tot} = J_{slow}$), it is possible to calculate the kinetic parameters for the lumped slow systems. Afterward, using the estimated parameters, this relationship may be used for the transient at Cf-NT or TG-TW (where $J_{tot} = J_{NCX} + J_{slow}$), which allows calculation of NCX parameters. Finally, using the same relationship for the twitch transient and the parameters already estimated, it is possible to estimate the SR-A parameters. It should be stressed that these parameters are empirical and do not necessarily correspond to those determinable directly in subcellular preparations. However, because this kinetic approach takes into account the relationship between transport velocity and $[Ca^{2+}]_i$, it is not necessary to work with Ca^{2+} transients of similar amplitudes.

By applying the calculated kinetic parameters for each transport system to the twitch $[Ca^{2+}]_i$ signal, individual Ca^{2+} fluxes were determined during twitch relaxation. The Ca^{2+} flux via each transporter, as well as the sum of all fluxes (J_{tot}), were integrated over one second after the peak of the twitch Ca^{2+} transient, and the percent contribution of each individual flux to J_{tot} was calculated. In this study, for each cell, NCX kinetic parameters were estimated using both Cf-NT and TG-TW, and each of these estimates was used to determine SR-A parameters and flux.

Fluxes are expressed as μM (i.e., μmol

Ca^{2+}/liter of cytosol). Data are presented as means ± SEM, or accompanied by the 95% confidence interval (95%CI). Comparison of data was performed by one-way analysis of variance for paired samples (followed, when necessary, by the Bonferroni test) or *t*-test for paired samples. Statistical significance was considered to occur when $P \leq 0.05$. Percentages were transformed to arc sin $\sqrt{p}$ prior to statistical comparisons; afterward, means and 95%CI limits were converted back to percentage. Prism (version 2.0, GraphPad Software, San Diego, CA, USA) was used for curve fitting and statistical analysis.

Figure 1A shows typical Ca^{2+} transients obtained in an isolated rat ventricular myocyte during control twitches, Cf-NT, TG-TW and Cf-00, and Figure 1B depicts mean values of diastolic $[Ca^{2+}]_i$, $\Delta[Ca^{2+}]_i$ and *K*. Diastolic $[Ca^{2+}]_i$ depended on the type of contraction ($P < 0.001$) and was lower for transients obtained after a 10- to 20-s rest (e.g., Cf-00 and TG-TW) than after a 2-s rest (Cf-NT and control twitches, $P < 0.05$). $\Delta[Ca^{2+}]_i$ also depended on the kind of transient ($P < 0.001$): caffeine-evoked transients were significantly higher than control twitches ($P < 0.05$), while TG-TW was lower than control twitches ($P < 0.001$). This is expected because the amount of Ca^{2+} released from the SR by caffeine is at least twice as large than that released during a twitch (17). Also, impairment of SR function by TG generally decreases Ca^{2+} transient amplitude in the mammalian ventricle (17,18), although, in the present experiments TG-TW amplitude was not so small because of the lower stimulation frequency (17). *K* was highly variable among types of contraction ($P < 0.001$), but comparable in Cf-NT and TG-TW ($P > 0.05$), during which SR Ca^{2+} accumulation is inhibited.

The total Ca^{2+} flux (integrated over 1 s after the twitch $[Ca^{2+}]_i$ peak) was slightly, but significantly ($P = 0.0072$) higher when TG-TW was used to estimate NCX-dependent Ca^{2+} flux (Table 1). These values are

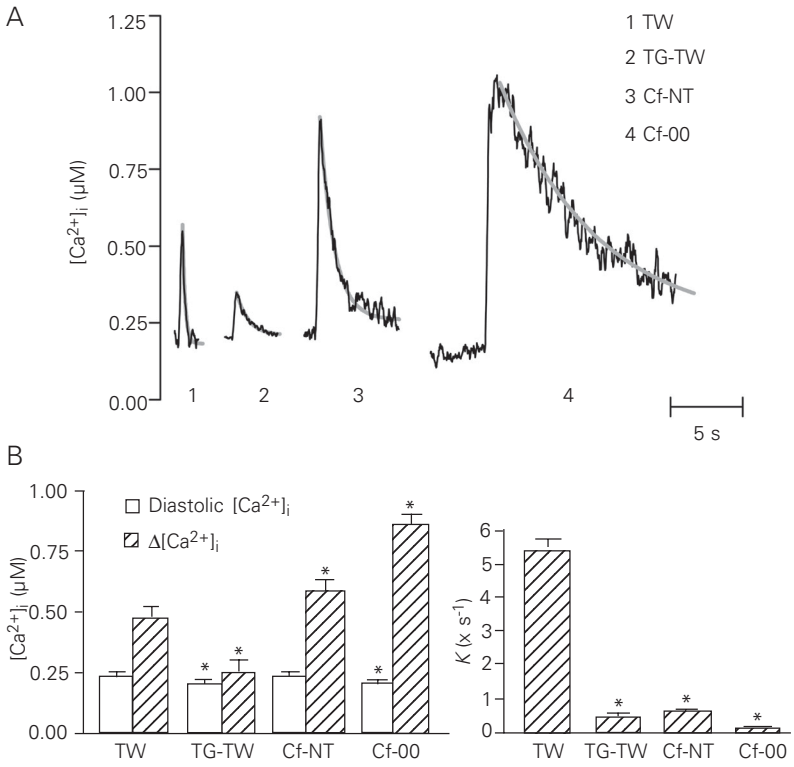

Figure 1. Ca^{2+} transients during inhibition of Ca^{2+} transporters. *A*, Typical Ca^{2+} transients recorded from an adult rat ventricular myocyte during electrical stimulation at 0.5 Hz under control conditions (TW) and at 0.1 Hz after treatment with thapsigargin (TG-TW), and in quiescence after application of 10 mM caffeine in the presence (Cf-NT) and absence (Cf-00) of extracellular Na^+ and Ca^{2+}. The gray curves superimposed on the traces represent single exponential functions fitted to the phase of $[Ca^{2+}]_i$ decline of each transient ($R^2 > 0.97$ in all cases). *B*, Values of diastolic $[Ca^{2+}]_i$ and Ca^{2+} transient amplitude ($\Delta[Ca^{2+}]_i$, left panel) and rate constant of $[Ca^{2+}]_i$ decline (right panel) for all four types of transient. *P < 0.05 compared to TW (one-way ANOVA for paired samples, followed by the Bonferroni test).

Table 1. Participation of the Na^+-Ca^{2+} exchanger (NCX) and the sarcoplasmic reticulum Ca^{2+}-ATPase (SR-A) in twitch relaxation.

	TG-TW	Cf-NT
Integrated Ca^{2+} flux (μM)		
Total	91.05 ± 6.78*	90.36 ± 6.86
NCX	12.06 ± 2.03*	7.10 ± 0.81
SR-A	76.68 ± 5.90*	80.96 ± 6.62
% Contribution to relaxation		
NCX	12.56 (10.81-14.43)*	7.92 (6.95-8.96)
SR-A	84.87 (82.85-85.96)*	89.48 (88.25-90.64)

Integrated Ca^{2+} fluxes (total, and carried by NCX and SR-A) estimated during twitch relaxation in isolated rat ventricular myocytes using caffeine application to quiescent cells (Cf-NT) or electrically evoked twitches after thapsigargin treatment (TG-TW), to inhibit SR Ca^{2+} accumulation. Data are reported as mean ± SEM. Mean relative contributions of NCX and SR-A to twitch relaxation are also shown, accompanied by the 95% confidence intervals in parentheses. N = 20. *P < 0.05 compared to Cf-NT (*t*-test for paired samples).

close to those previously described for adult rat myocytes during a twitch (3,7). The use of TG-TW resulted in a higher estimate of the NCX-dependent integrated Ca^{2+} flux ($P = 0.0314$) and a lower estimate of SR-A-dependent Ca^{2+} flux ($P = 0.0491$) compared to the use of Cf-NT (Table 1). The integrated flux carried by the slow transporters was determined independently of NCX and amounted to 2.30 ± 0.22 μM.

As a result of the differences in individual Ca^{2+} fluxes, the relative contribution of each Ca^{2+} transport system to twitch relaxation showed statistically significant differences depending on whether TG-TW or Cf-NT was used to estimate NCX participation. The contribution of the slow systems was not significantly affected (2.6% of the total integrated flux with both TG-TW and Cf-NT). However, estimated NCX participation was greater with TG-TW than that with Cf-NT ($P = 0.0174$). Conversely, SR-A contribution was lower with TG-TW than with Cf-NT ($P = 0.0169$), as shown in Table 1. The relative contributions of NCX and SR-A to twitch relaxation obtained in the present study with Cf-NT were comparable to those previously described using the same approach in adult rat ventricular myocytes (3,7).

The present results thus show that using TG-TW instead of Cf-NT to selectively inhibit SR Ca^{2+} accumulation significantly increased the estimated contribution of NCX to cytosolic Ca^{2+} clearance associated with twitch relaxation. In a previous study we had also compared Cf-NT and TG-TW and observed, as seen in the present analysis, that the time course of $[Ca^{2+}]_i$ decline was similar in both types of transients; however, Ca^{2+} fluxes were not determined in that study (3). Moreover, in the previous analyses (3,4,7-9), Ca^{2+} fluxes and the relative contributions of Ca^{2+} transporters to relaxation were estimated with average Ca^{2+} transient data, which yielded a single value for a given cell population. In the present study, we analyzed data from individual cells, which not only permitted us to estimate the variability within the cell population, but also permitted us to submit the data to statistical comparison.

Using Cf-NT to selectively inhibit SR Ca^{2+} accumulation is less costly and much easier, from an experimental point of view, than using TG-TW. However, the use of TG-TW presents some advantages, such as: a) differently from caffeine, TG directly inhibits its SR-A; b) during TG-TW, the Ca^{2+} transient is triggered by an action potential waveform, although it has been shown that TG treatment decreases the action potential duration in rat ventricular myocytes (18); c) because the TG-TW transient has much lower amplitude than that obtained with Cf-NT, the NCX-mediated inward current and the likelihood of undesirable changes in V_m are considerably smaller. On the other hand, the marked effect of SR inhibition in depressing the Ca^{2+} transient amplitude might pose a problem with TG-TW because of the difficulty of extracting reliable data from a low-amplitude signal. This can be partly overcome by decreasing the stimulation frequency, as done in the present study. However, it is important that one be certain that full inhibition of SR-A is obtained with TG treatment because incomplete blockade greatly affects the results and renders them unreliable.

Although the mean difference in estimated NCX-mediated Ca^{2+} flux and NCX relative contribution to the $[Ca^{2+}]_i$ decline during twitch relaxation reached as much as 50% when data obtained with Cf-NT and TG-TW were compared, only in 9 out of 20 cells were these values more than 20% higher with TG-TW. This suggests that caffeine might have caused V_m changes in only part of the cell population studied. Despite the present results, caffeine might still be considered a valuable tool in this kind of experiment, as long as its limitations are acknowledged. Also, care should be exercised when using Cf-NT for this type of analysis under conditions that favor cell Ca^{2+} overload (12) and/or reduce resting V_m stability, such as down-

regulation of the inward rectifying K^+ current (I_{K1}) that may occur in some kinds of heart disease (20), which are likely to facilitate the undesirable effects of caffeine on V_m.

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