Estimation of the fractional sarcoplasmic reticulum Ca^{2+} release in intact cardiomyocytes using integrated Ca^{2+} fluxes

Rosana A. Bassani[1], Rafael A. Ricardo[2] and José W. M. Bassani[1,2]

[1] *Center for Biomedical Engineering, University of Campinas, Campinas, Sao Paulo, Brazil*
[2] *Department of Biomedical Engineering, School of Electrical and Computer Engineering, University of Campinas, Campinas, Sao Paulo, Brazil*

Abstract. The sarcoplasmic reticulum (SR) is the main source of contraction-activating Ca^{2+} in the adult mammalian myocardium. The fraction of the SR Ca^{2+} content released at a twitch (fractional SR Ca^{2+} release, FR) is an important parameter for assessing the efficiency of excitation-contraction coupling under physiological and pathophysiological conditions, as well as for identification of modulators of this process. We here describe and propose an approach for FR quantitation based on the estimation of integrated Ca^{2+} fluxes mediated by different transporters that remove the ion from the cytosol. These fluxes may be calculated solely from the measurement of cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) during Ca^{2+} transients evoked under selective inhibition of the transporters, and from the cell Ca^{2+} buffering parameters available in the literature. The FR values obtained with this approach in intact rat ventricular myocytes (0.63 ± 0.04; $n = 12$) were comparable to those estimated in the same cell type with an already established method, based on electrophysiological measurements with the patch-clamp technique, in addition to $[Ca^{2+}]_i$ measurement (0.69 ± 0.05; $n = 6$; $p > 0.40$). We conclude that the proposed method might be a suitable and a technically simpler alternative to the electrophysiological method for FR estimation.

Key words: Sarcoplasmic reticulum — Fractional calcium release — Excitation-contraction coupling — Myocardium

Abbreviations: $[Ca^{2+}]_i$, free Ca^{2+} concentration; $[Ca^{2+}]_T$, total Ca^{2+} concentration; FR, fractional sarcoplasmic reticulum Ca^{2+} release; $I_{Ca,L}$, L-type Ca^{2+} current; NCX, Na^+/Ca^{2+} exchanger; RyR, ryanodine receptor; SR, sarcoplasmic reticulum; SERCA, sarco-endoplasmic reticulum Ca^{2+}-ATPase; TG, thapsigargin; TS, Tyrode´s solution.

Introduction

The sarcoplasmic reticulum (SR) is the main source of the Ca^{2+} that activates contraction in mammalian myocardium. During the excitation-contraction coupling process, Ca^{2+} that enters the cell *via* sarcolemmal currents binds to Ca^{2+} channels in the SR membrane (also known as ryanodine receptors, RyR), triggering SR Ca^{2+} release (Bers 2002). The resulting increase in cytosolic free Ca^{2+}

concentration ($[Ca^{2+}]_i$) not only allows contraction to develop, but also enhances the transport rate of the systems that remove Ca^{2+} from the cytosol, of which the most important are the SR Ca^{2+}-ATPase (SERCA, which refills the SR store) and the Na^+/Ca^{2+} exchanger (NCX, which mediates transarcolemmal Ca^{2+} efflux). The remaining transporters, namely the sarcolemmal Ca^{2+}-ATPase and the mitochondrial Ca^{2+} uniporter, contribute less than 3% to the $[Ca^{2+}]_i$ decline associated with relaxation (Bassani et al. 1992, 1994).

The SR does not release all of its Ca^{2+} content at a twitch. The fraction of this content that is released (known as fractional SR Ca^{2+} release, FR) is not necessarily constant, and is an important determinant of the efficiency of the

Correspondence to: Rosana A. Bassani, Centro de Engenharia Biomédica, UNICAMP, R. Alexander Fleming 163, 13083-881 Campinas, Sao Paulo, Brazil
E-mail: rosana@ceb.unicamp.br

excitation-contraction coupling process (Bassani et al. 1993). FR is tightly regulated by the amplitude of the L-type Ca^{2+} current ($I_{Ca,L}$), the main trigger of Ca^{2+} release, as well as by the SR Ca^{2+} content (Bassani et al. 1995a; Shannon et al. 2000; Ginsburg and Bers 2004). In addition, local modulation of RyRs may affect the relationship between FR and the trigger Ca^{2+} and/or $[Ca^{2+}]_{SR}$ (Li et al. 1997; Kohlhaas et al. 2006).

Available evidence suggests that FR may be altered in several pathophysiological conditions, such as arterial hypertension, tachyarrhythmia, myocardial ischemia and heart failure (e.g., Delbridge et al. 1997; Gómez et al. 1997; Shorofsky et al. 1999; Sah et al. 2001; Carvalho et al. 2006; Heinzel et al. 2008; Lenaerts et al. 2009), and in some transgenic models in which Ca^{2+} homeostasis is disturbed (Maier et al. 2003; Gusev et al. 2009; Sedej et al. 2010), as well as during aging (Dibb et al. 2004). However, quantitation of FR in isolated cardiomyocytes is not often performed, mostly due to the limitations and/or complexity of the available methodological approaches. Currently there are two main approaches: the twitch depletion (Bassani et al. 1993) and the electrophysiological methods (Ginsburg and Bers 2004). Although these methods are apparently reliable for FR quantitation and result in comparable values, both present disadvantages: while the former is not applicable to steady-state conditions, the latter is more technically complex and time-consuming.

The goal of the present study was to develop an alternative approach for FR determination, which may be used during steady-state electric stimulation of intact myocytes, without the need of electrophysiological measurements. The proposed approach, based on the calculation of integrated Ca^{2+} fluxes, resulted in FR estimates comparable to those obtained with the electrophysiological method.

Materials and Methods

Myocardial preparations

Myocytes were isolated from the left ventricle of adult male Wistar rats by collagenase digestion during retrograde perfusion at 37°C (Penna and Bassani 2010). Rats were maintained at 23 ± 2°C, under a 12 h:12 h light-dark cycle, and had free access to food and water. Euthanasia was performed by exsanguination following cerebral concussion. Animal care and euthanasia protocols were in accordance to the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (1986), and were approved by the institutional Committee of Ethics in Animal Research (CEUA/IB/UNICAMP, protocol numbers P1517-1 and P1737-1).

Cells were plated on a collagen-coated perfusion chamber placed on the stage of an inverted microscope, and perfused with modified Tyrode's solution (TS; composition in mM: 140 NaCl, 6 KCl, 1 $CaCl_2$, 1 MgCl, 5 HEPES, 11 glucose; pH 7.4) at 23°C. Except for the electrophysiological experiments, electric field stimulation (rectangular, symmetric, biphasic voltage pulses, 5 ms duration, 1.2 × threshold amplitude, 0.5 Hz) was delivered through a pair of platinum electrodes.

For $[Ca^{2+}]_i$ measurement, myocytes were incubated with the fluorescent Ca^{2+} indicator indo-1 AM (Molecular Probes, Eugene, OR, USA; 5 µM) for 15 min, and then perfused with TS for 30 min for indo-1 washout. Indo-1 was excited at 360 nm. A computerized system consisting of a microfluorimeter (Photon Technology International, Monmouth Junction, USA) coupled to an epifluorescence microscope was used to control indo-1 excitation, as well as for recording the emitted fluorescence at two wavelengths (410 and 485 nm). The ratio (R) of the background-subtracted emission at 410 and 485 nm was converted to $[Ca^{2+}]_i$ according to the equation:

$$[Ca^{2+}]_i = K_d \cdot \beta \, [(R - R_{min})/R_{max} - R)] \tag{1}$$

where R_{min} and R_{max} are R values at minimal (0 mM $CaCl_2$ + 7 mM EGTA) and saturating $[Ca^{2+}]$ (20 mM $CaCl_2$ + 7 mM EGTA), respectively, determined in isolated myocytes. β was determined according to Gomes et al. (1998) and indo-1 K_d was 0.844 µmol/l (Bassani et al. 1995b).

Determination of Ca^{2+} fluxes

The experimental protocol is shown in Figure 1. Shortly, cells were field-stimulated for 3 min, then electric stimulation was interrupted for a few seconds until $[Ca^{2+}]_i$ attained a stable level. Stimulation was resumed for 2 min, and interrupted again. Cells were then perfused with 0 Na-0 Ca solution (with composition similar to TS, except for equimolar replacement of NaCl and $CaCl_2$ with LiCl and EGTA, respectively) for 20–30 s, followed by perfusion with the same solution containing 15 mM caffeine, to cause the SR to release all of its Ca^{2+} content (Bassani et al. 1992). After caffeine washout, cells were incubated with 10 µM thapsigargin (TG), an irreversible SERCA inhibitor (Sagara et al. 1992). The effectiveness of the TG treatment at preventing SR Ca^{2+} reloading was assessed by application of caffeine following 2 min-long electrical stimulation. Cells that responded to caffeine with any visible $[Ca^{2+}]_i$ elevation were discarded. The next step was to stimulate the cells with a 3 min-long stimulus train, as done before TG treatment.

Ca^{2+} fluxes during the decline phase of a Ca^{2+} transient were estimated according to Bassani et al. (1994), with
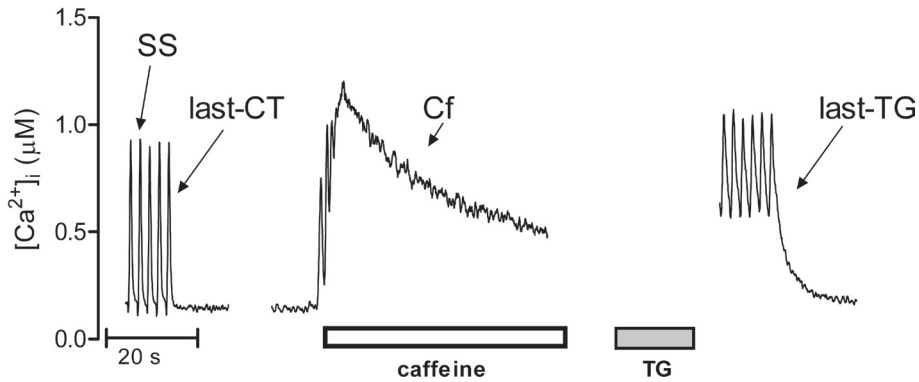
Figure 1. [Ca^{2+}]$_i$ traces recorded from rat ventricular myocytes, showing the sequence of experimental maneuvers required for determination of the fractional sarcoplasmic reticulum (SR) Ca^{2+} release with the Ca^{2+} flux approach. The protocol consisted of electrical stimulation for 3 min in control conditions, during which Ca^{2+} transients at steady-state twitches (SS, i.e., after 3 min electrical stimulation), as well as the twitch evoked by the last electric stimulus (last-CT) were recorded. Afterward, the SR Ca^{2+} content was fully released by sustained caffeine application in Na$^+$, Ca^{2+}-free solution (transient labeled as Cf). The cell was then treated with 10 µM thapsigargin (TG) for 2 min, and the 3 min-long stimulus train was repeated. The twitch evoked by the last stimulus of this train (last-TG) was also recorded. For more details, see text.

modifications. Briefly, the total flux of Ca^{2+} removal from the cytosol (J_{tot}) was assumed to be:

$$J_{tot} = J_{SR} + J_{NCX} + J_{slow} \qquad (2)$$

where J_{SR}, J_{NCX} and J_{slow} are the Ca^{2+} fluxes carried by SERCA, NCX and the combination of the slow transporters (i.e., sarcolemmal Ca^{2+}-ATPase and mitochondrial Ca^{2+} uniporter), respectively. J_{tot} was estimated during the decay phase of 3 types of transient, in which different sets of transporters were active. These transients are shown in Figure 1:

a) the last twitch of the 3-min long stimulus train under control conditions, i.e., before TG treatment (last-CT): all 3 systems contribute to cytosolic Ca^{2+} removal;

b) the last twitch of the 3-min long stimulus train, applied after TG treatment (last-TG): in this case, [Ca^{2+}]$_i$ decline should rely only on NCX and the slow systems, as SERCA was completely inhibited.

c) the transient evoked by 10 mM caffeine (Cf): during the decline of this transient, the SR is unable to accumulate Ca^{2+} because the sustained application of caffeine maintains the RyRs leaky, thus emptying the SR Ca^{2+} store (Bassani et al 2004). In addition, NCX is thermodynamically inhibited by the absence of extracellular Na$^+$ and Ca^{2+}, so that cytosolic Ca^{2+} clearance can be attributed solely to the slow transporters. This transient was also used for estimation of the SR Ca^{2+} content (see below).

For each type of transient, the free [Ca^{2+}] signal (i.e., [Ca^{2+}]$_i$) was converted to total [Ca^{2+}] ([Ca^{2+}]$_T$, i.e., the sum of the free and bound [Ca^{2+}]) by:

$$[Ca^{2+}]_T = [Ca^{2+}]_i + \{B_{max-en} \cdot [Ca^{2+}]_i/(K_{d-en} +$$
$$+ [Ca^{2+}]_i)\} + \{B_{max-in} \cdot [Ca^{2+}]_i/(K_{d-in} + [Ca^{2+}]_i)\} \qquad (3)$$

where B_{max-en} and K_{d-en} are the maximal concentration and apparent dissociation constant of high-affinity, endogenous passive Ca^{2+} binding sites (300 and 0.53 µM, respectively, Bassani et al. 1998); B_{max-in} and K_{d-in} are the parameters related to indo-1 (considered as 20 and 0.844 µM, respectively; Bassani et al. 1995b).

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

$$J_x = V_{max} / \{1 + (K_m/[Ca^{2+}]_i)^n\} \qquad (4)$$

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

Applying this relationship to the decline of the Cf transient (in which $J_{tot} = J_{slow}$), it is possible to calculate the kinetic parameters for the combined slow transporters. Using the latter parameters and equations 2 and 4, it is possible to estimate the NCX parameters using the last-TG transient ($J_{tot} = J_{NCX} + J_{slow}$). The procedure was then repeated for the last-CT transient, for calculation of SERCA parameters. Finally, the parameters of the 3 transporters were used to estimate the individual Ca^{2+} fluxes during a regular transient under steady-state stimulation (SS, see Figure 1). The [Ca^{2+}]$_i$ data used for flux estimation during the SS twitch were the average values of 3 successive

transients just before the last. The fluxes were integrated during the period from the transient peak to the time of attainment of diastolic $[Ca^{2+}]_i$. This analysis was performed for each of the 12 studied cells.

Electrophysiological measurements

Membrane potential (V_m) and current were measured in the whole-cell patch-clamp configuration (preamplifier mod. HL-1-17; amplifier mod. Axopatch 2B, Axon Instruments, Inc., Foster City, CA, USA). Pipettes were made from borosilicate tubes (TW150-3, World Precision Instruments Inc., Sarasota, USA) and filled with either of the following solutions (composition in mM): a) 130 potassium glutamate; 7 NaCl; 10 HEPES; 0.5 EGTA; pH 7.2 (V_m measurement); and b) 130 CsCl; 5 MgCl$_2$; 1 KCl; 10 HEPES; 1 EGTA; pH 7.2 (current measurement). The resistance of the solution-filled pipette was 1–3 or 7–8 MΩ, for voltage- and current-clamp experiments, respectively.

Electrophysiological measurements were carried out on indo-1-loaded myocytes. After establishment of the gigaseal between the membrane and the pipette tip, negative pressure was applied to allow rupture of the membrane patch and electrical access to the cell. V_m was held at –85 mV, and membrane capacitance was estimated from the temporal decay of the capacitive current evoked by a rectangular pulse to –90 mV. Current and voltage signals were acquired at 10 kHz and filtered at 5 kHz.

Action potentials (AP) were evoked by stimulation with rectangular current pulses (6 nA, 2 ms duration) at 0.5 Hz. The liquid junction potential at the pipette tip was compensated for in the measurements, which were performed in 8 cells. A representative AP waveform was selected to be used as a template for voltage stimulation (AP-clamp; Bouchard et al. 1995; Bassani et al. 2004), to better simulate the AP developed under electric field stimulation.

For current measurements, V_m was held at –85 mV, and the cell was stimulated under AP-clamp. Cell capacitance and series resistance were partially compensated (60–80%). The L-type Ca^{2+} current was measured after switching the perfusate to the $I_{Ca,L}$ solution, designed to block contaminating ion currents (composition in mM: 140 tetraethylammonium chloride; 3 4-aminopyridine; 4 CsCl; 1 MgCl$_2$; 10 HEPES; 1 CaCl$_2$; pH 7.4). Before exposure to this solution, cells were preperfused with 0 Na-0 Ca solution for 10 min for depletion of intracellular Na$^+$, thus minimizing Na$^+$ influx *via* NCX in the presence of the Na$^+$-free, Ca^{2+}-containing $I_{Ca,L}$ solution (Bassani et al. 1992). Preliminary experiments with rectangular voltage pulses indicated that $I_{Ca,L}$ could be successfully isolated with this experimental protocol. After 10 conditioning AP-clamp pulses were applied at 0.5 Hz

(sufficient to reload the SR with Ca^{2+}, Bassani et al. 2004), $I_{Ca,L}$ was recorded at the 11th pulse, simultaneously with the Ca^{2+} transient. Afterward, cells were exposed to 0 Na-0 Ca solution containing 10 mM caffeine, for estimation of the SR Ca^{2+} content.

Estimation of the fractional SR Ca^{2+} release

Ca^{2+} flux approach

We assumed that, at steady-state, NCX extrudes a similar amount of Ca^{2+} that enters the cell during excitation (mainly *via* $I_{Ca,L}$), and that the amount of Ca^{2+} taken up by the SR during the decline of the transient is equivalent to that released from the organelle during excitation-contraction coupling. Thus, the latter amount would be equivalent to $\int J_{SR}\, dt$ during the SS twitch. Previous studies support the validity of such an assumption (Delbridge et al. 1996; Trafford et al. 2002). FR at the SS twitch was considered as the ratio of $\int J_{SR}\, dt$ and the SR Ca^{2+} content.

Electrophysiological approach

The initial step was to calculate the change in $[Ca^{2+}]_T$ due to Ca^{2+} influx *via* $I_{Ca,L}$ ($\Delta[Ca^{2+}]_{T,\,ICa,L}$), estimated as:

$$\Delta[Ca^{2+}]_{T,ICa,L} = \frac{\int I_{Ca,L} dt}{z \cdot F \cdot Vc \cdot f_{nm}} \tag{5}$$

where $\int I_{Ca,L}\, dt$ is the integrated current over the duration of the voltage stimulus; Vc is the cell volume estimated from cell length and width (measured with an ocular micrometer), assuming cylindrical cell shape (Vornanen 1996); f_{nm} is the non-mitochondrial fraction of cell volume (0.65); z and F are the ion valence and the Faraday constant, respectively. The amount of Ca^{2+} released from the SR at the test transient was calculated as the difference of the peak variation in $[Ca^{2+}]_T$ and $\Delta[Ca^{2+}]_{T,ICa,L}$. FR was then considered as the ratio of the amount of released Ca^{2+} and the SR Ca^{2+} load (Ginsburg and Bers 2004).

In both approaches, the SR Ca^{2+} content was estimated as the difference in $[Ca^{2+}]_T$ values at the peak of the transient evoked by caffeine and at diastole, immediately before caffeine application (Bassani et al. 1995a; Carvalho et al. 2006).

Data analysis

The decay phase of the Ca^{2+} transients was fitted by a monoexponential function for determination of the time constant (τ). Integrated fluxes and $[Ca^{2+}]_T$ values are expressed as μM (μmol Ca^{2+} *per* liter of non-mitochondrial cell water). Data are shown as means ± standard error. Student´s *t* test

for paired or unpaired was used for comparison of the data obtained with the two approaches. $p < 0.05$ was considered as the limit for statistical significance.

Results

At steady-state stimulation, field-stimulated ($n = 12$) and voltage-clamped myocytes ($n = 6$) presented similar values of diastolic $[Ca^{2+}]_i$ (0.21 ± 0.02 and 0.20 ± 0.02 μM, respectively) and transient amplitude (0.58 ± 0.06 and 0.55 ± 0.06 μM, respectively; $p > 0.62$).

$[Ca^{2+}]_i$ decline during the last-CT twitch was longer than during the SS twitch (τ: 0.192 ± 0.011 vs. 0.159 ± 0.006 s; $p < 0.01$), probably because $[Ca^{2+}]_i$ declined to a lower diastolic value in the former (0.18 ± 0.01 vs. 0.21 ± 0.02 μM; $p < 0.01$, t test for paired samples). The mean amplitude of the declining phase of the transients used for determination of the kinetic parameters of the transporters was: last-CT: 0.61 ± 0.06 μM; last-TG: 0.36 ± 0.06 μM; and Cf: 1.18 ± 0.10 μM. The lower amplitude of the last-TG transient and the greater amplitude of the Cf transient, compared to last-CT, are expected because during the former the SR should not be able to release Ca^{2+}, whereas the latter was generated by release of all the SR Ca^{2+} content. As observed previously (Bassani et al. 1992, 1994), the fewer Ca^{2+} transporters remained active during $[Ca^{2+}]_i$ decline, the greater was the τ value: last-CT: 0.192 ± 0.011 s; last-TG: 1.70 ± 0.10 s; and Cf: 14.7 ± 1.4 s.

The calculated empirical parameters of the transporters are presented in Table 1.

In the electrophysiological experiments, cell capacitance was 137 ± 12 pF, and estimated cell volume was 31.4 ± 5.7 pl. The mean AP data from the measurements under current-clamp were: diastolic membrane potential: –81.0 ± 3.6 mV;

Table 1. Empirical kinetic parameters for Ca^{2+} transport systems, estimated from $[Ca^{2+}]_i$ data obtained from isolated rat ventricular myocytes ($n = 12$)

|	SERCA	NCX	Slow systems
V_{max} (μM/s)	292 ± 19	26.6 ± 3.2	4.54 ± 0.38
K_m (μM)	0.31 ± 0.02	0.33 ± 0.02	0.32 ± 0.02
n	3.83 ± 0.15	5.54 ± 0.41	3.62 ± 0.10

Data are presented as mean ± standard error. K_m, $[Ca^{2+}]_i$ at which the transport velocity is half-maximal; n, Hill coefficient; NCX, Na$^+$/Ca^{2+} exchanger; SERCA, sarcoplasmic reticulum Ca^{2+} ATPase; slow systems, combination of sarcolemmal Ca^{2+} ATPase and mitochondrial Ca^{2+} uniporter; V_{max}, maximum velocity of transport.

AP amplitude: 124 ± 5 mV; AP duration at 90% repolarization: 89 ± 11 ms. At the 11th AP clamp stimulus, $I_{Ca,L}$ peak density was –3.30 ± 0.37 pA/pF, and whereas the time integral of $I_{Ca,L}$ was 17.2 ± 2.6 pC.

Figure 2A depicts the mean values of Ca^{2+} influx (considered as $\int J_{NCX}\,dt$ and $\Delta[Ca^{2+}]_{T,ICa,L}$ in the Ca^{2+} flux and electrophysiological approaches, respectively) and amount of the ion released from the SR at a twitch, estimated with both methods, as well as the SR Ca^{2+} content. Method-dependent, significant difference was not observed for any of these estimates ($p > 0.19$). The FR values calculated, shown in Figure 2B, were similar with both approaches ($p > 0.40$).

Discussion

We here propose an alternative method for FR estimation in intact cardiomyocytes, based on calculation of Ca^{2+} fluxes mediated by different transporters during the decline of
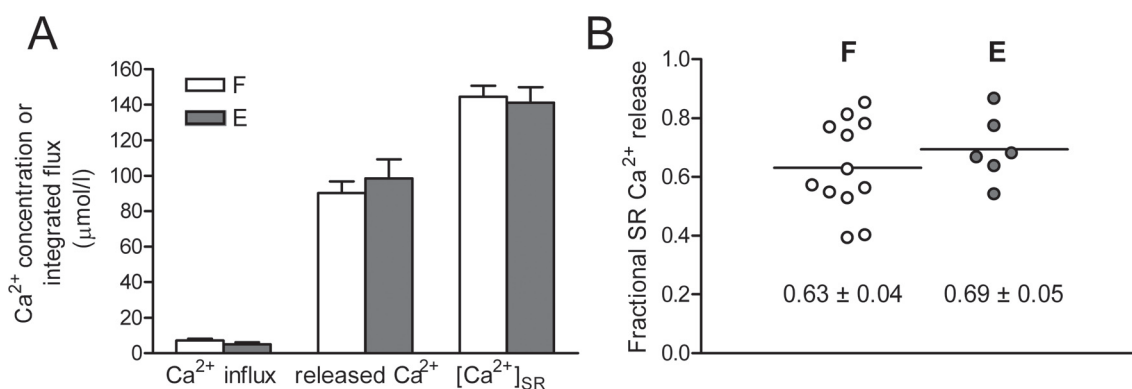
Figure 2. A. Estimates of the total $[Ca^{2+}]$ (i.e., free + bound) variation due to Ca^{2+} influx and of the amount of Ca^{2+} released from the sarcoplasmic reticulum (SR) at a twitch, as well as of the SR Ca^{2+} content ($[Ca^{2+}]_{SR}$) determined in isolated rat ventricular myocytes using the proposed Ca^{2+} flux approach (F) and the electrophysiological method (E). **B.** The fraction of the SR Ca^{2+} content released at a twitch estimated with both methods; the mean values are shown below the points, and indicated by the horizontal lines. The number of studied cells was 12 and 6 for the F and E methods, respectively.

Ca^{2+} transients in which different sets of transporters are active. Estimation of these fluxes has been used to assess the relative contribution of these transporters for twitch relaxation in several conditions (Bassani et al. 1994; Li et al. 1997; Bassani and Bassani 2002; Piacentino et al. 2003; Carvalho et al. 2006; Curran et al. 2007), as well as for the estimation of SERCA-mediated Ca^{2+} flux during spontaneous Ca^{2+} release (MacQuaide et al. 2007) and of SERCA empirical kinetic parameters in intact cells (Picht et al. 2007; Bassani et al. 2012). Fluxes can be easily calculated from $[Ca^{2+}]_i$ measurements and from Ca^{2+} buffering parameters available in the literature (Hove-Madsen and Bers 1993; Berlin et al. 1994; Bassani et al. 1998; Trafford et al. 1999), provided that the signal of the Ca^{2+} indicator is properly calibrated.

FR is an important parameter for assessment of the efficiency of excitation-contraction coupling, as most of the Ca^{2+} pool available for myofilament activation is released from the SR in the adult mammalian heart. The first method proposed for FR quantitation, the twitch depletion method (Bassani et al. 1993), allows FR determination in intact cells and is technically straightforward. Briefly, it consists in the estimation of the SR Ca^{2+} content after a single test twitch following SERCA inhibition with TG. Nevertheless, this method is not applicable to steady-state conditions, as a minimum period of 2 min is required for TG treatment before the test twitch. Thus, factors that may affect FR and are dependent on the duration of the diastolic interval (e.g., time-dependent recovery of ion channel availability, Ca^{2+}-dependent activity of enzymes) cannot be investigated. Moreover, because of the rest interval required for TG incubation, FR may be overestimated if the experimental treatment enhances spontaneous diastolic SR Ca^{2+} release.

The electrophysiological method, described by Ginsburg and Bers (2004) and also used in this report, is based on the determination of $[Ca^{2+}]_T$ variation during a twitch-associated Ca^{2+} transient, from which the contribution of Ca^{2+} influx *via* $I_{Ca,L}$ is subtracted. Because it does not require drug treatment before the test twitch, this approach is suitable for application during steady-state stimulation. On the other hand, the requirement of reliable $I_{Ca,L}$ measurement makes experiments technically more complicated, with a low rate of success. In addition, depending on the pipette resistance and on the composition of the filling solution, FR might be affected by intracellular perfusion, which may modify modulation of RyR activity by endogenous factors.

In the present study, we estimate that in rat ventricular myocytes, the SR releases ~65% of its content during a twitch at steady-state, cyclic activity. This value is not far from those previously described in rabbit, ferret, mouse and rat ventricular myocytes (~50–60%), in which either the twitch depletion (Bassani et al. 1993, 1995a; Delbridge et al. 1996) or the electrophysiological method (Shannon et al. 2000; Ginsburg and Bers 2004) was used. Moreover, the FR values estimated with the

Ca^{2+} flux approach were comparable to those determined with the electrophysiological method under similar experimental conditions. Similarity of the FR values estimated with both methods was also observed under experimental conditions in which variation of $I_{Ca,L}$ amplitude was accompanied by changes in FR (Ricardo R. A., Bassani R. A. and Bassani J. W. M., unpublished results). These observations indicate that the proposed approach allows reliable quantitation of this parameter, with the advantage of requiring simpler experimental protocol and apparatus.

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