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Estimation of Ca^{2+} recirculating fraction in rat ventricle: influence of the methodological approach

Estimação da fração de recirculação de Ca^{2+} em ventrículo de rato: influência da abordagem experimental

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Abstract
Ca^{2+} recirculating fraction (RF) has been determined in isolated myocardium as an estimate of the fraction of activator Ca^{2+} that circulates between cytoplasm and sarcoplasmic reticulum (SR) during activity. RF is represented by the slope of the linear relationship between the amplitude of successive contractions (B_1, B_2...B_n) during decay of twitch potentiation. In this study, we induced potentiation by rest (the most frequently employed method) and by rapid pacing in isolated ventricular muscle from adult and developing rats. Measurements were taken at 1 Hz stimulation rate. In adults, the relationship B_n *vs.* B_{n+1} was significantly linear and RF values were similar (~0.92) with both methods. In young hearts, this relationship was significantly linear when rapid pacing was used, but poor linearity was observed with the rest-potentiation approach. RF increased with development ($P<0.01$), which is in agreement with post-natal maturation of cardiac SR. However, RF values were much lower ($P<0.01$) with the rest approach (~0.1-0.4 over the first three post-natal weeks), compared with rapid pacing (~0.6-0.8). Our results suggest that, whereas reliable RF estimates can be obtained with rapid pacing, care should be taken when using the rest approach to determine RF in conditions that might affect the interval-force relationship, such as post-natal development and cardiovascular disease.
Keywords: Cell Ca^{2+} cycling, Myocardium, Post-natal development, Sarcoplasmic reticulum, Twitch-potentiation.

Resumo
A fração de recirculação de Ca^{2+} (RF) determinada em miocárdio isolado tem sido considerada uma estimativa da fração do Ca^{2+} ativador da contração que circula entre o citoplasma e o retículo sarcoplasmático (SR) durante a atividade. RF é representada pelo coeficiente angular da reta que descreve a relação entre as amplitudes de contrações sucessivas (B_1, B_2...B_n) durante a extinção de potenciação contrátil. Neste estudo, induzimos potenciação por pausas estimulatórias (o método mais frequentemente utilizado) e por aumento da frequência estimulatória em músculo ventricular isolado de ratos adultos e em desenvolvimento. A tensão isométrica foi medida durante estimulação a 1 Hz. Em adultos, a relação B_n vs. B_{n+1} foi significativamente linear e os valores de RF foram semelhantes (~0.92) com ambos os métodos. Em corações de ratos imaturos, esta relação foi linear quando potenciação foi induzida por aumento de frequência, mas nem sempre quando o método de potenciação por pausa foi usado. RF aumentou com a idade ($P<0.01$), como esperado, considerando-se a maturação pós-natal do SR. No entanto, os valores de RF foram bem menores ($P<0.01$) com o uso de pausa (~0.1-0.4 durante as primeiras 3 semanas após o nascimento), do que com a indução de potenciação por aumento da frequência estimulatória (~0.6-0.8). Nossos resultados sugerem que esta última abordagem permite estimativas confiáveis de RF, e apontam a necessidade de cautela ao utilizar o método da pausa para determinar RF em condições que afetem a relação força-intevalo, como, por exemplo, o desenvolvimento pós-natal e patologias cardiovasculares.
Palavras-chave: *Ciclagem celular de Ca^{2+}, Potencialização da contração.*

Introduction

The sarcoplasmic reticulum (SR) is the main source of contraction-activating Ca^{2+} in the mammalian heart. At each beat, Ca^{2+} entering the cell during the action potential *via* sarcolemmal Ca^{2+} channels triggers SR Ca^{2+} release, which causes cytoplasmic Ca^{2+} concentration to increase and, thus, contraction to develop (Bers, 1991). During relaxation, Ca^{2+} is removed from the cytoplasm mainly by the SR Ca^{2+}-pump (thus, restoring SR Ca^{2+} stores to be released at the next beat), while a lesser amount of Ca^{2+} is extruded via the Na^{+}-Ca^{2+} exchange (NCX) (Bassani *et al.*, 1992, 1994; Negretti *et al.*, 1993). Thus, during rhythmic contractile activity, Ca^{2+} cycles predominantly in the intracellular environment (i.e., it recirculates between cytoplasm and SR). The fraction of Ca^{2+} that undergoes intracellular recirculation during subsequent beats (Ca^{2+} recirculating fraction, RF) has been empirically estimated in isolated cardiac preparations with basis on the relative decline of the amplitude of successive contractions during dissipation of twitch potentiation (e.g., Mörner and Wohlfart, 1992; Vornanen, 1992). That is, the amplitude of the contraction after a potentiated beat (during which SR Ca^{2+} release is considered to be massive) will depend on the amount of previously released Ca^{2+} which returned to the SR during relaxation of the potentiated twitch, and is available for further release (i.e., on how much Ca^{2+} recirculates between the two beats).

The myocardium of newborn mammals exhibits signs of SR immaturity, in the morphological, biochemical and functional aspects (see Mahony, 1996). Thus, it has been proposed that, in neonates, Ca^{2+} cycling between the cytoplasm and the extracellular medium is more prominent than in adults, and that the proportion of activator Ca^{2+} that cycles between cytoplasm and SR increases as the SR matures. However, the extent of this change has not been ascertained yet (see, e.g., Vornanen, 1992; Bassani & Bassani, 2000).

In the present study, we estimated RF in ventricular muscle from adult and developing rats, using two different methods to produce potentiation of electrically-evoked contractions (twitches). We observed that the RF values obtained in the developing muscle were strongly dependent on the method employed in RF determination, and that rapid pacing-induced potentiation yielded more reliable RF estimates.

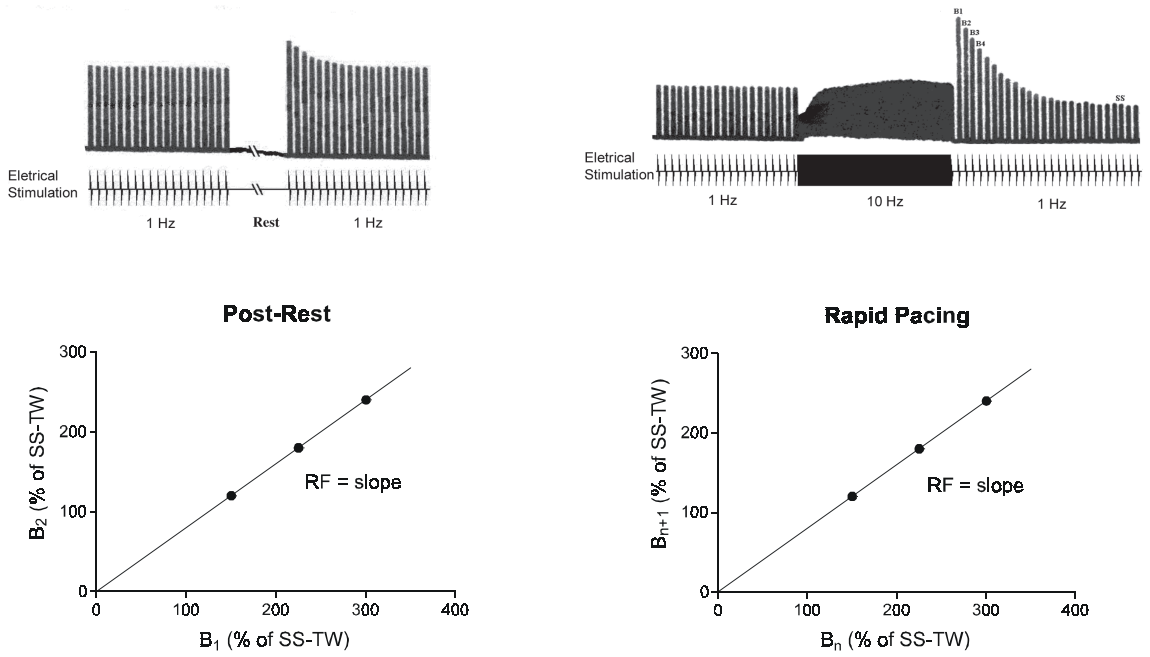

Figure 1. Procedure to determine RF in isolated ventricular muscle, in which twitch potentiation is induced by: prolonged rest (left panels) and rapid pacing at 10 Hz (right panels). Original isometric tension traces recorded during each protocol are depicted (upper panels). The relationship between the normalized amplitude of successive twitches is shown in the lower panels (see text for details). Observe that the points shown in the graphs are illustrative, and do not represent experimental results.

Methods

Papillary muscles and wall strips were obtained from the right ventricles of Wistar rats with ages ranging between 1 day and 3 months (adults). The muscle was perfused with Krebs-Henseleit solution (129.3 mM NaCl, 4.6 mM KCl, 1.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 10.7 mM $NaHCO_3$, 1.2 mM $MgSO_4$ and 11.1 mM glucose, pH 7.4 at 36.5 °C under 95% O_2 - 5% CO_2 gassing) and electrically stimulated at 1 Hz (bipolar voltage pulses of amplitude 1.2x threshold and 2 ms duration). Active tension was measured with an isometric force transducer (Narco Biosystems, mod. F60). After 40 min equilibration at the optimal muscle length, RF was determined. Experiments were conducted on 5 preparations of each age. Two methods were used to achieve twitch potentiation (see Figure 1).

a) rest: the rat ventricle responds to prolonged diastole with potentiation of the first post-rest twitch (B_1). In this approach, different rest periods were applied to each preparation (30-600 s), after which stimulation at 1 Hz was resumed. For each rest duration, the amplitude of the second post-rest twitch (B_2) was plotted as a function of the amplitude of the preceding twitch (B1, i.e., the first twitch evoked after rest). The amplitude of all post-rest twitches was converted to the percentage of that of twitches under steady-state stimulation at 1 Hz (SS-TW, i.e., the average amplitude of the last 5 twitches before each rest interval was applied). A straight line was then fitted to the experimental points and RF was estimated as the resulting slope.

b) rapid pacing: the muscle was stimulated at 10 Hz for 20 s, after which stimulation rate was dropped back to 1 Hz. Decrease in stimulation rate caused a transient increase in twitch amplitude, which waned out after a few beats. Considering only the negative staircase phase at 1 Hz, after decreasing stimulation rate, the amplitude of successive beats was plotted as a function of that of the preceding beat (B_2 *vs*. B_1, B_3 *vs*. B_2, and so on) over a range of 4-6 beats. Again, amplitude was normalized to that of the SS-TW at 1 Hz, and the slope of the linear relationship was considered to be RF.

Data, expressed as means ± standard error (SEM), were compared by two-way analysis of variance and *post-hoc* t-test. A P value ≤ 0.05 was taken as indicative of statistical significance.

Results

Figure 2 shows the relationship between the amplitude of a given twitch and that of the preceding contraction using rest (panel A) and rapid pacing (panel B) approaches.

RF values determined with the post-rest potentiation method were 0.10 ± 0.08, 0.21 ± 0.03, 0.39 ± 0.05 and 0.92 ± 0.03 in muscles from rats aged 1-6, 7-14, 15-22 and 90 days-old, respectively (N = 5 for all age groups) . Using the rapid pacing potentiation method in the same preparations, RF values were 0.60 ± 0.04, 0.69 ± 0.04, 0.85 ± 0.07 and 0.93 ± 0.06 for the same age groups, respectively. Analysis of variance revealed significant age-dependence of RF values ($P< 0.01$). However, absolute RF values were also strongly dependent on the method used to induce twitch potentiation ($P< 0.01$). With the rest method, RF values obtained in developing rats at all ages were different from that in adults (i.e., 90 days-old, $P< 0.05$), whereas with the rapid pacing method, RF values were higher than with the rest approach, and significantly lower than in adults solely until the end of the second postnatal week, with attainment of mature values at age 3 weeks (see Figure 3).

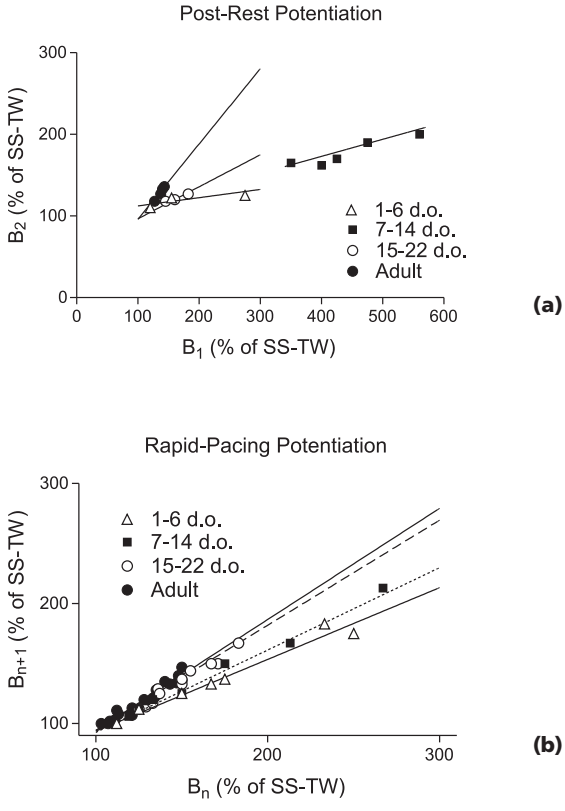

Figure 2 – Estimated Ca^{2+} recirculating fraction in ventricular muscle from adult and developing rats, determined by the rest **(a)** and the rapid pacing **(b)** approaches.

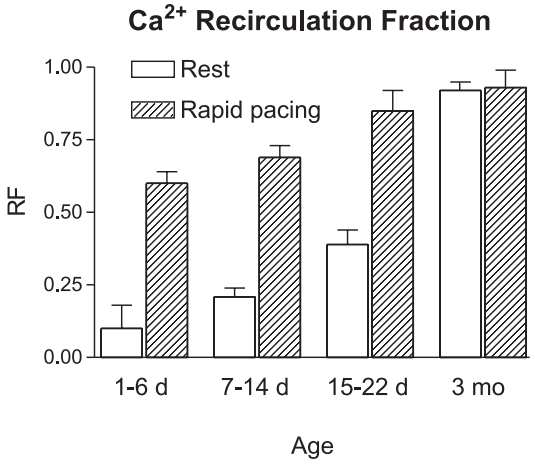

Figure 3. RF values determined in adult and developing ventricle, estimated by the rest and rapid pacing approaches. Bars are mean values and vertical lines represent SEM values (N=5).

In adult muscle, both methods yielded similar RF values, and the relationship of the amplitudes of successive twitches was significantly linear ($r^2 > 0.95$). However, in preparations from developing animals, the relationship between B_2 and B_1 amplitudes determined with the rest approach showed poor linearity, especially in younger preparations ($r^2 = 0.07$, 0.74 and 0.76 in 1-, 2- and 3-week old rats, respectively). On the other hand, using the rapid-pacing approach, the relationship between B_{n+1} and B_n was significantly linear at all ages ($r^2 > 0.87$, $P < 0.05$). These results indicate that, whereas in adult ventricle both methods seem to be comparable, in immature ventricular muscle the post-rest approach does not seem adequate for RF determination.

Discussion

The recirculating fraction of Ca^{2+} (RF) determined in isolated cardiac muscle is thought to represent an estimate of the fraction of contraction-activating Ca^{2+} that recirculates in the intracellular environment between successive beats. At steady-state, it is assumed that the total amount of cell Ca^{2+} is constant. Thus, the same amount of Ca^{2+} released by the SR during a given beat should be taken up back to this organelle during relaxation. Likewise, Ca^{2+} influx during the action potential should equal Ca^{2+} efflux during relaxation. This assumption has received compelling experimental evidence (Delbridge *et al.*, 1996; 1997; Yuan *et al.*, 1996).

In adult rat ventricular myocytes, we have estimated that the SR Ca^{2+}-pump is responsible for ~90% of the Ca^{2+} fluxes associated with relaxation (Bassani *et al.*, 1994). Pharmacological suppression of SR function has also shown to depress contraction amplitude by 90% in this preparation (Bers, 1985; Kirby *et al.*, 1992), which gives support to the proposal that approximately 90% of the activator Ca^{2+} recycles inside the cell during repeated activity. Accordingly, the total amount of Ca^{2+} entering the cell during an action potential-like depolarization waveform matches the amount of Ca^{2+} extrusion estimated during relaxation of the same cell type (~ 7-9 % of total Ca^{2+} fluxes, Bassani *et al.*, 1994; Yuan *et al.*, 1996; Delbridge *et al.*, 1997). The RF determined in multicellular preparations from adult rats in the present study (~92%) was similar to the estimate of the relative contribution of the SR Ca^{2+} pump in isolated cells, as described above. This suggests that, although using mechanical activity as the measured variable (the amplitude of which shows a non-linear relationship with cytoplasmic Ca^{2+} concentration), RF determination may be useful to evaluate the contribution of the SR Ca^{2+} pool to contraction. However, one should keep in mind the limitations of the use of RF. For instance, interval-dependent changes in excitation-contraction coupling, which have been demonstrated to occur in cardiac cells (see, e.g., Bers *et al.*, 1993), are not taken into account in RF estimation.

Vornanen (1992) also determined RF in ventricles from adult and developing rats, using rest to induce twitch potentiation. His estimates (from 0.11 to 0.65 over the 3 first post-natal weeks, and 0.97 in adults) were comparable to those obtained by us with the same experimental approach. However, we observed that the decay of twitch amplitude after rest potentiation was not linear in developing myocardium (i.e., proportional amplitude decay between B_2 and B_1 was not similar after different rest periods) . This non-linearity precludes the use of rest-induced potentiation in the determination of RF in immature hearts, although this method appeared to be adequate for adult preparations. Ontogenetic differences in the influence of the preceding interval on SR Ca^{2+} release might give rise to non-linearity of the B_2-B_1 relationship. Alternatively, rest-dependent changes in NCX function might modify the competition between the SR Ca^{2+}-pump and the exchanger during relaxation of the first post-rest twitch, leading to different RF depending on rest duration. This would be particularly important in immature ventricles, in which NCX ge-

netic expression and function have been reported to be greater than in adult preparations (Boerth *et al.* 1994; Bassani & Bassani 2000). This problem might also occur with other preparations which show altered force-interval relationship, such as ventricular myocardium from animals with cardiac hypertrophy and arterial hypertension (Baudet *et al*., 1992; Perez *et al*., 1993), and/or which show NCX overexpression, such as the hypertrophic ventricle (O'Rourke *et al*., 1999; Pogwizd *et al*., 1999).

With the use of an alternative approach, namely rapid pacing, significant linearity was observed in all types of preparation. This might be due to the fact that, with the rest approach, the intervals preceding B_1 and B_2 are markedly different: while the latter was always 1 s, the former varied between 30 and 600 s. However, with rapid pacing, interval-related variations of twitch amplitude decay are not expected to affect the B_{n+1} *vs*. B_n relationship, since all considered contractions are evoked after a 1-s stimulatory interval. Although none of the approaches allows measurement under steady-state conditions, rapid pacing-potentiation probably yields more reliable RF estimates. Interestingly, the RF values here reported in developing and adult rat myocardium are comparable to the relative contribution of the SR to relaxation-associated Ca^{2+} fluxes (0.6-0.85), determined in isolated myocytes of rats at the same ages (Bassani and Bassani, 2000).

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