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SHORT COMMUNICATION

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A novel, rapid and reversible method to measure Ca buffering and time-course of total sarcoplasmic reticulum Ca content in cardiac ventricular myocytes

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Abstract This paper outlines a simple method of estimating both the Ca-buffering properties of the cytoplasm and the time-course of changes of sarcoplasmic reticulum (s.r.) Ca concentration during systole. The experiments were performed on voltage-clamped ferret single ventricular myocytes loaded with the free acid of fluo-3 through a patch pipette. The application of caffeine (10 mM) resulted in a Na-Ca exchange current and a transient increase of the free intracellular Ca concentration ($[Ca^{2+}]_i$). The time-course of change of total Ca in the cell was obtained by integrating the current and this was compared with the measurements of $[Ca^{2+}]_i$ to obtain a buffering curve. This could be fit with a maximum capacity for the intrinsic buffers of 114±18 µmol l^{-1} and K_d of 0.59±0.17 µM (n=8). During the systolic rise of $[Ca^{2+}]_i$, the measured changes of $[Ca^{2+}]_i$ and the buffering curve were used to calculate the magnitude and time-course of the change of total cytoplasmic Ca and thence of both s.r. Ca content and Ca release flux. This method provides a simple and reversible mechanism to measure Ca buffering and the time-course of both total cytoplasmic and s.r. Ca.

Key words Ca buffering · Calcium · Sarcoplasmic reticulum

Introduction

The introduction of fluorescent indicators has facilitated the measurement of the free cytoplasmic Ca concentration ($[Ca^{2+}]_i$) in cardiac cells. However, in order to relate Ca measurements to Ca fluxes it is important to know the time-course and magnitude of changes of *total* rather than free Ca. This requires knowledge of the Ca buffering. One approach is to calculate the expected Ca buffering from the known concentrations of various ligands. This gives reasonable estimates of the increase of $[Ca^{2+}]_i$ produced by known entries of Ca [7]. Ca buffering can be measured readily in permeabilized cells [4] from the changes of free Ca produced by known additions of total Ca. It is harder to measure in intact cells because part of the added total Ca will be removed by transport across both sarcolemmal and s.r. membranes. This problem has been circumvented by inhibiting Ca-removal mechanisms [2]. Under these conditions, depolarizing pulses to different voltages can be used to add known amounts of Ca to the cell and the resulting increase of $[Ca^{2+}]_i$ measured. This method suffers from the fact that the s.r. inhibition (with thapsigargin) is irreversible and therefore cells cannot be used subsequently for other studies.

Therefore, work using this method has generally taken buffering measurements obtained from one group of cells and used them for experimental studies on other cells. The initial aim of the work described here was to produce a simpler and reversible method for characterizing buffering power. We show that it is possible to construct a buffering curve from a single application of caffeine to a voltage-clamped cell.

Sarcolemmal Ca entry can be measured, under voltage-clamp, from the L-type Ca current (upon depolarization) and the Ca efflux from the electrogenic Na-Ca exchange current (upon repolarization) [5, 8]. However, it is more difficult to measure the s.r fluxes and changes of s.r. Ca content. The s.r. Ca content can be measured under *steady-state* conditions by adding caffeine to release it and integrating the resulting current as the Ca is pumped out of the cell on Na-Ca exchange [9]. This method, however, gives no information about changes of s.r. Ca *during* systole. In the present work, we make use of the measured properties of cellular Ca buffers, to estimate changes of *total* cytoplasmic Ca from the changes of $[Ca^{2+}]_i$ during systole. After correction for sarcolemmal Ca fluxes, it is therefore possible to estimate the net Ca flux across the s.r. membrane and thence the changes of s.r. Ca content.

Materials and methods

The experiments were performed on ventricular myocytes isolated from ferrets killed by an overdose of sodium pentabarbitone (i.p.) as described previously [8]. Voltage-clamp control was imposed with the whole-cell technique. A switch clamp (Axoclamp 2B, Axon Instruments, Foster City, Calif., USA) was used at switching rates of 4–25 kHz. $[Ca^{2+}]_i$ was measured using the free acid form of fluo-3 loaded via the patch pipette. All data were corrected for a 14-mV junction potential. Experiments were carried out at room temperature (23°C). Na-Ca exchange currents were integrated and corrections made for non Na-Ca exchange fluxes, as described

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previously, in order to estimate total Ca fluxes [5, 8]. Estimates of s.r. Ca content and sarcolemmal fluxes are related to total cell volume. Values are presented as the mean ±SEM of data from *n* cells.

The superfusing solution contained (mM): NaCl, 140; glucose, 10; HEPES, 10; KCl, 6; $CaCl_2$, 2; $MgCl_2$, 1; titrated to pH 7.4 with NaOH. The pipette solution contained (mM): K-glutamate, 120; KCl, 30; HEPES, 10; $MgCl_2$, 5; KATP, 5; Na_2-creatine phosphate, 3.6; NaCl, 2.8; cAMP, 0.05; Fluo-3$(K)_5$, 0.025; pH 7.2 with KOH.

Calibration of fluo-3 records

Movement artefacts were avoided by measuring fluorescence from the entire cell and ensuring that illumination intensity was uniform. Fluo-3 has the property that fluorescence is negligible in the absence of Ca. Therefore,

$$F=F_{max}[Ca^{2+}]_i/([Ca^{2+}]_i+K_d) \quad (1)$$

where F is observed fluorescence and F_{max} the maximum fluorescence. In much previous work F_{max} has been calculated by assuming a value for the resting Ca concentration [3]. In the present work, however, we have measured F_{max} directly by damaging the cell with the patch pipette and measuring the increase of fluorescence. A value of 400 nM was used for the K_d.

Results

Determination of the Ca-buffering curve

In the experiment illustrated in Fig. 1A, caffeine was applied to release Ca from the s.r. This resulted in a transient increase of $[Ca^{2+}]_i$ which was accompanied by an inward electrogenic Na-Ca exchange current as Ca^{2+} ions were pumped out of the cell. We have analysed this by integrating the current backward in time. In this direction $[Ca^{2+}]_i$ increases with time as does the integral caused by Na-Ca exchange. If Na-Ca exchange is the only mechanism which pumps $[Ca^{2+}]_i$ out of the cell then the change in the integral of the current between two time points would give a measure of the amount of Ca expelled from the cell to change $[Ca^{2+}]_i$ between two levels. However, the integral has to be corrected for non Na-Ca exchange fluxes. This corrected integral then gives a measure of the total amount of Ca required to change $[Ca^{2+}]_i$ over the observed range. This relationship (shown in Fig. 1B) effectively gives a measure of the Ca buffering of the cell. The relationship cannot be determined for values of $[Ca^{2+}]_i$ below the resting value. The parameters of the buffer are therefore obtained by fitting the curve with an arbitrary offset (a). Although this may result in error in the determination of these parameters, it is important to realize that it does not affect the accuracy of the relationship between *changes* of free and total Ca. In this experiment the buffering could be characterized with a maximum capacity (B_{max}) of 109 µmol l^{-1} and a K_d of 0.60 µM. The mean total buffering curve is shown in Fig. 1C. In general the data were well fit by this buffering curve (mean r^2=0.98±0.005). This buffering represents the sum of: (1) the intrinsic Ca buffering of the cell and (2) the buffering provided by the added fluo-3. To calculate the latter we assume [2] that 65% of the cell volume is cytoplasm and therefore that the 25 µM fluo-3 in the pipette corresponds to 16 µM when referenced to *total* cell volume. The solid line in Fig. 1C represents the total Ca buffering and the dashed line the calculated contribution from fluo-3; therefore, subtracting this from the total gives the intrinsic buffering (dotted line) having a mean B_{max} of 114±18 µmol l^{-1} and a K_d of 0.59±0.17 µM (n=8).

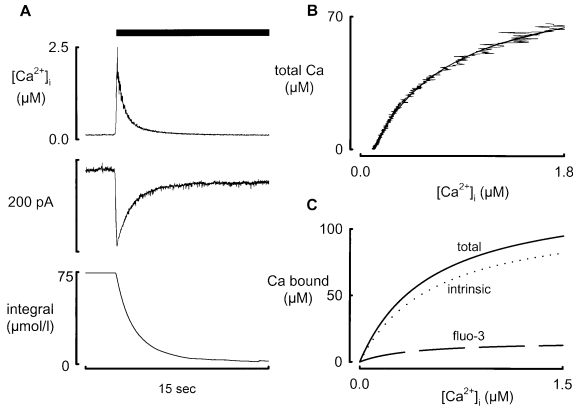

Fig. 1A–C Determination of intracellular Ca buffering. **A** Caffeine (10 mM) was applied as shown (*solid bar*). *Traces* show (*top* to *bottom*): $[Ca^{2+}]_i$; membrane current; cumulative integral: the baseline for this integral is set to zero at the end of the trace for use in determining the buffering properties in **B**. **B** The relationship between free (*abscissa*) and total (*ordinate*) Ca. The *noisy trace* is the raw data replotted from **A** and the smooth trace is a fit to: total $Ca=a+\{B_{max}[Ca^{2+}]_i/(K_d+[Ca^{2+}]_i)\}$. **C** Mean data from 8 cells showing the total buffering, that due to fluo-3 and the calculated intrinsic buffering (subtracting the fluo-3 component from the total). Membrane potential was –40 mV throughout

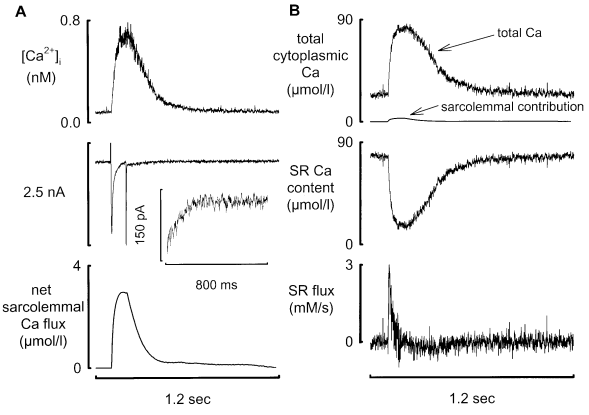

Fig. 2A, B Estimation of total Ca movements. **A** (*Top* to *bottom*): $[Ca^{2+}]_i$; membrane current produced by a 100-ms-duration pulse from –40 to 0 mV; *inset* shows an amplified version of the Na-Ca exchange tail current on repolarization; calculated change of cell Ca content due to sarcolemmal Ca fluxes. **B** (*Top* to *bottom*): calculated total cytoplasmic Ca (sarcolemmal contribution indicated); sarcoplasmic reticulum (*s.r.*) Ca content; release flux from s.r.

Time-course of total cytoplasmic and s.r. Ca

In the experiment of Fig. 2, the cell was depolarized with a voltage-clamp pulse. Figure 2A shows the resulting Ca transient (top) and membrane currents (middle). The top trace of Fig. 2B shows the calculated changes in total cytoplasmic Ca using the buffering parameters obtained from the same cell. In this case the increase of free Ca was 700 nM whereas that of total Ca was 63 µM. The sarcolemmal fluxes were estimated by integrating the membrane currents [5, 8]. This resulted in a calculated increase of total Ca of about 3 µmol l^{-1} during the Ca current and the loss of an identical amount of Ca via Na-Ca exchange on repolarization (bottom panel, Fig. 2A). Plotting this on the same scale

as the total Ca transient (top Fig. 2B) makes it clear that the sarcolemmal fluxes are much smaller than those of total Ca. When these changes of total Ca due to sarcolemmal fluxes are subtracted from the total Ca transient then the result gives the amount of Ca that must be released across the s.r. membrane to produce the observed changes in $[Ca^{2+}]_i$. The diastolic value of s.r. Ca was obtained from a caffeine application (not shown) and found to be 76 µmol l^{-1} (total cell volume). With this knowledge the time-course of intra-sarcoplasmic reticular Ca can be calculated. In this case s.r. Ca decreases from 76 to 15 µmol (l cell)$^{-1}$ (Fig. 2B, middle). This method allows one to calculate the flux of Ca from the s.r. by differentiating the s.r. Ca content with respect to time. The bottom panel of Fig. 2B shows that the peak rate of Ca efflux from the s.r. increases the total cytoplasmic Ca at a rate of 3 mmol l^{-1} s^{-1}. This value is in good agreement with that reported previously using a different method [7].

Discussion

This paper presents a simple method to characterize the Ca-buffering properties of the cell and makes use of this to calculate changes of total cytoplasmic and s.r. Ca content and the underlying fluxes. Before discussing these data, it is important to consider the accuracy of the method used.

The method requires calculating changes of *total* from measurements of *free* Ca concentration. This is done by measuring the amount of Ca pumped out of the cell during the decay of the caffeine-evoked response as a function of $[Ca^{2+}]_i$. We assume that, during exposure to caffeine, the only way in which total cytoplasmic Ca can change is as a result of fluxes across the cell membrane. This requires that: (1) s.r. Ca release is over very early in the caffeine-evoked response and (2) mitochondria do not contribute significantly to Ca buffering. We also assume that, at least on the time-course of changes of $[Ca^{2+}]_i$ during the caffeine response, bound and free Ca are equilibrated (see [2] for discussion). It should also be noted that, although caffeine shifts the relationship between $[Ca^{2+}]_i$ and tension towards lower $[Ca^{2+}]_i$, this does not affect Ca binding and therefore buffering [6]. As in previous work [5, 8, 9] we have to correct for the fact that some of the Ca is pumped out of the cell by the electroneutral Ca-ATPase (see Materials and methods). Another potential problem is that the measurements of $[Ca^{2+}]_i$ are obtained from a non-ratiometric Ca indicator (fluo-3). Any calibration errors will not matter for many purposes. Thus in the determination of the magnitude of the total Ca transient and s.r. Ca content, all that matters is that a given fluo-3 fluorescence signal corresponds to a given $[Ca^{2+}]_i$.

The present method used to study buffering can be compared with those used previously. The most rigorous study to date was of permeabilized rabbit myocytes where complications caused by sarcolemmal fluxes could be avoided [4]. Buffering in intact cells has been studied by measuring the rise of $[Ca^{2+}]_i$ produced by a given amount of Ca entry into the cell as measured from the integral of the L-type Ca current. The values obtained in such studies have been used to estimate changes of s.r. Ca content from changes in the magnitude of the rise of $[Ca^{2+}]_i$ produced by caffeine [1]. This method suffers from two problems.

1. As mentioned in the Introduction, the requirement to inhibit the s.r. Ca-ATPase makes it irreversible and therefore work using this approach has generally used mean values of buffering obtained from one group of cells to analyse the buffering in other cells.
2. Allowance must be made for the (often unknown) buffering produced by the Ca indicator in the cell.

In contrast the present method requires only one exposure to the completely reversible compound caffeine. Since it is performed on the cell under study, buffering of any added indicators is automatically included. Furthermore, if the aim is only to calculate the time-course of total systolic Ca (rather than characterize the buffering), the present method does not even require that the measured fluorescence be calibrated in absolute units of $[Ca^{2+}]_i$. It would be adequate to obtain the relationship between fluorescence and total Ca during a caffeine-evoked response and use this to calculate changes of total Ca from those of fluorescence during a systolic Ca transient. Finally, the present method gives results that reasonably agree with those obtained previously. For example, one previous study of intact cells [2] found that the intrinsic buffers have a Bmax of 123 µmol l–1 accessible cell water and a K_d of 0.96±0.18 µM. Work on permeabilized cells [4] found a Bmax of 215 µmol l–1 accessible cell water and a K_d of 0.42 µM for the higher affinity buffer. Assuming that 65% of the cell volume is non-mitochondrial water then our Bmax equates to 175 µmol l–1 accessible cell water, a value which is intermediate between those reported above. Similarly the K_d is also in between the previous values.

We conclude that the present method provides a simple technique for characterizing cellular Ca buffering and measuring the fluxes of Ca across the s.r. membrane.

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