Stimulation of Ca-induced Ca release only transiently increases the systolic Ca transient: measurements of Ca fluxes and sarcoplasmic reticulum Ca

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Received 4 August 1997; accepted 19 September 1997

Abstract

Objective: To investigate the effects of stimulating calcium induced Ca release with low concentrations (100–200 μM) of caffeine and, in particular, to study the cellular mechanisms responsible for the transient responses found previously. Methods: Experiments were performed on isolated rat ventricular myocytes. Intracellular calcium concentration [Ca] was measured with Indo-1, the cells were voltage-clamped with the perforated patch technique and sarcoplasmic reticulum (s.r.) Ca content was estimated from the integral of the caffeine-evoked current. Results: The systolic Ca transient produced by the first depolarization in the presence of caffeine was larger than the control. Over the next few pulses the magnitude of the Ca transient returned to control levels despite the maintained presence of caffeine. The s.r. Ca content was decreased by 9% after one pulse in caffeine and by 21% after several pulses in caffeine. The first pulse in the low concentration of caffeine was followed by an enhanced inward (Na–Ca exchange) current tail indicating increased efflux of calcium from the cell. The extra loss of calcium calculated from the tail current agreed quantitatively with that from the change of s.r. Ca content. Conclusions: These results show that stimulating calcium induced calcium release produces only a transient increase of the systolic Ca transient. This is due to the larger Ca transient decreasing the s.r. Ca content. It is concluded that any agent whose sole mode of action is stimulation of calcium-induced calcium release will not produce a maintained inotropic effect. The consequences of this for the effects of other modulators of calcium induced calcium release are discussed. © 1998 Elsevier Science B.V.

Keywords: Calcium-induced calcium release; Sarcoplasmic reticulum; Calcium; Rat; Systole

1. Introduction

It is now generally accepted that a major mechanism initiating calcium release from the sarcoplasmic reticulum (s.r.) in the heart is calcium-induced calcium release (CICR; for review, see [1]). Here Ca entry through the sarcolemmal L-type currents initiates opening of the s.r. Ca channels (ryanodine receptors) leading to the release of s.r. Ca. Given this background, there is considerable interest in whether the magnitude of the systolic rise of [Ca^{2+}], can be controlled by regulating the properties of calcium induced calcium release. For example, it has recently been suggested that the compound cyclic ADP ribose (cADP-Ribose) may stimulate CICR and hence increase the systolic Ca transient [2–4]. In addition the release channel is known to be controlled by phosphorylation and application of phosphatases has been shown to decrease the gain of calcium-induced calcium release [5–7]. Of clinical relevance is the fact that some immunosuppressant drugs such as rapamycin or FK-506 may bind to an FK binding protein associated with the ryanodine receptor and thence lead to a stimulation of Ca release [8,9]. Furthermore it has recently been suggested that impairment of CICR may account for the decreased systolic Ca transient in cardiac hypertrophy [10].

In contrast to the work suggesting that modulation of calcium-induced Ca release will affect systolic calcium, we have previously suggested that this modulation will have
only a transient effect. Thus potentiation of CICR with low (sub millimolar) concentrations of caffeine produced only a transient increase in the magnitude of the systolic Ca transient [11]. We suggested that the transient nature of the response arose because the stimulation of calcium-induced calcium release decreases the s.r. Ca content and this exactly offsets the potentiation of CICR. In that original study, however, there were no quantitative measurements of either s.r Ca content or the sarcolemmal fluxes which may produce these changes of s.r. content. It has recently become possible to measure the s.r. Ca content [12] and also to relate changes of s.r. Ca content to fluxes across the sarcolemma [13,14]. The aim of the present paper was therefore to answer the following questions: (i) Is the effect of caffeine on systolic [Ca$^{2+}$], transient accompanied by a decrease of s.r. Ca content? (ii) Is the decrease of s.r. content quantitatively sufficient to account for the decrease of systolic Ca transient back toward control levels? (iii) Can the decrease of s.r. Ca content be accounted for by measured sarcolemmal fluxes?

2. Methods

2.1. Experimental techniques

The investigation conforms with the ‘Guide for the Care and Use of Laboratory Animals’ published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1985). Experiments were performed on single cardiac myocytes isolated from the ventricles of rats using a collagenase and proteinase digestion technique as previously described [15]. Isolated myocytes were loaded with the [Ca$^{2+}$], sensitive fluorescent indicator Indo-1 as the cell permeant acetoxymethyl ester (2.5 μM for 5 min, followed by at least 30 min deesterification) and placed in a bath (volume 125 μl) on the stage of an inverted microscope adapted for epi-fluorescence (Nikon Diaphot 300, Nikon UK). Fluorescence was excited at 400 nm and measured at 400 and 500 nm, the ratio of the emitted fluorescence (400:500) was used as an index of [Ca$^{2+}$], [16].

2.2. Voltage clamp

Voltage clamp control was achieved using the perforated patch technique [17] with amphotericin-B. To compensate for the relatively high access resistance of the perforated patch technique the switch clamp facility of the Axoclamp-2B (Axon Instruments Inc. CA, USA) patch clamp amplifier was used at switching rates of 1–4 kHz. The resting membrane potential was held at −40 mV and 100 ms depolarizing voltage clamp pulses to 0 mV were applied at 0.33 Hz. Electrodes were fabricated from borosilicate glass and had resistances of 1–3 MΩ when filled with (mM): KCl, 125; K$_{2}$SO$_{4}$, 125; KCl, 20; NaCl, 10; HEPES, 10; MgCl$_{2}$, 5; titrated to pH 7.2 with KOH. Amphotericin-B was prepared as a stock solution in DMSO (60 mg ml$^{-1}$) and added to a final concentration of 240 μg ml$^{-1}$.

2.3. Solutions

Cells were bathed in a control solution of the following composition (mM): NaCl, 135; glucose, 11; HEPES, 10; KCl, 4; MgCl$_{2}$, 1.2; CaCl$_{2}$, 1; pH 7.4 with NaOH. To avoid interference from outward currents all experiments were performed in the presence of 5 mM 4-aminopyridine and 0.1 mM BaCl$_{2}$. All experiments were performed at 23°C.

2.4. Quantification of Sr Ca$^{2+}$ content and sarcolemmal Ca$^{2+}$ fluxes

The inward Na$^{+}$–Ca$^{2+}$ exchange currents produced by the application of 10 mM caffeine to a voltage clamped cell were integrated and converted to total calcium fluxes as described previously [12–14]. Net movements of Ca during and following voltage clamp pulses were calculated by integrating membrane currents [14]. The Na$^{+}$–Ca$^{2+}$ exchange currents were first corrected for the activity of non-electrogenic Ca$^{2+}$ extrusion pathways. Briefly, one needs to correct for the fact that, during a caffeine response, some of the calcium is removed from the cytoplasm by mechanisms other than Na–Ca exchange and does not, therefore, generate current. The magnitude of this correction was estimated as follows. The rate constant of decay of the caffeine response was measured (i) under control conditions ($k_{cont}$) and (ii) with the Na–Ca exchange inhibited with 10 mM Ni$^{2+}$ ($k_{Ni}$). Multiplying a measured Na–Ca exchange flux by $k_{cont}/(k_{cont}−k_{Ni})$ gives the corrected total Ca flux. This correction was used for the Na–Ca exchange fluxes activated by either caffeine or on repolarization (tail currents). The multiplying factor used was 1.5 [12]. Cell volume was calculated from the membrane surface area obtained from the membrane capacitance assuming a membrane capacitance/volume ratio of 6.76 pF pl$^{-1}$ for the rat [18].

2.5. Statistics

All values are presented as mean ± standard error of the mean for n cells.

3. Results

3.1. Effects of maintained caffeine application on Ca transient and s.r. content

Fig. 1 shows the effects of applying caffeine (200 μM) on a voltage-clamped myocyte. In agreement with previous
work [11], this results in a small transient increase of the systolic Ca transient with no effect on diastolic [Ca\(^{2+}\)]. The s.r. Ca content was also measured both before adding caffeine and after the transient increase of systolic Ca transient had decayed back to the steady level. This was done by adding a high (10 mM) concentration of caffeine to discharge the s.r. Ca content. The integral of the resultant Na–Ca exchange current then gives the magnitude of the s.r. Ca content. In the experiment illustrated in Fig. 1, the application of a low caffeine concentration decreased the s.r. Ca content from 68 to 59 \(\mu\)mol l\(^{-1}\). This effect was reversible after removing the low concentration of caffeine (s.r. Ca content of 73 \(\mu\)mol l\(^{-1}\)).

### 3.2. Sarcolemmal fluxes responsible for the changes of s.r. content

In experiments such as that shown in Fig. 2 we have investigated the origin of the decrease of s.r. Ca content produced by low concentrations of caffeine. Specifically, we have tested the hypothesis that the increased systolic rise of [Ca\(^{2+}\)] leads to stimulation of Na–Ca exchange and therefore to a greater loss of calcium from the cell. A low concentration (100 \(\mu\)M) of caffeine was applied and then removed. The application of caffeine again produced a transient increase of systolic [Ca\(^{2+}\)], and its removal a transient undershoot in the magnitude (cf. [11]). Specimen Ca transients are shown in more detail in the upper trace of Fig. 2B. The accompanying current records show a L-type Ca current on depolarization which is very slightly depressed during the first stimulus in caffeine. On repolarization there is an inward Na–Ca exchange current tail [19,20] which can be seen more clearly (a) in the inset. The first transient in the presence of caffeine is accompanied by a much larger Na–Ca exchange tail (b). In contrast the small first transient after removing caffeine has a smaller tail (c) which then recovers in the steady-state (d). The bottom traces show cumulative integrals of sarcolemmal Ca movements calculated as described in Section 2.2. In the initial and final steady-state records (a and d) there is an initial gain of Ca via the L-type current of about 4 \(\mu\)mol l\(^{-1}\) followed by an exactly equivalent loss on repolarization. In contrast, enhancing Ca-induced Ca release (b) leads to a net loss of calcium of about 5 \(\mu\)mol l\(^{-1}\) due to the increased Na–Ca tail current. Conversely, during the post-caffeine undershoot (c) the decreased Na–Ca current produces a net gain of calcium of 1.8 \(\mu\)mol l\(^{-1}\).

The above data show that low concentrations of caffeine decrease the s.r. Ca content as measured directly by the integral of the caffeine-evoked current. On average \((n = 9)\) the maintained application of 100 \(\mu\)M caffeine resulted in a significant reduction of the s.r. Ca content from 91 \(\pm\) 6 to 73 \(\pm\) 5 \(\mu\)mol l\(^{-1}\) \((P < 0.00002,\) paired \(t\)-test). Furthermore, the measured currents associated with
depolarization predict, qualitatively, a decrease of cell Ca content. By summing the net calculated losses of Ca from the cell on each pulse in the presence of low concentrations of caffeine it is possible to predict the net loss of Ca from the cell and compare this with that measured directly by the response to 10 mM caffeine. The histogram in Fig. 3 shows that the predicted Ca losses calculated from the net Ca movements on each pulse account for a large fraction of the measured loss. On average \((n = 9)\) the predicted loss of Ca was 69 ± 5% of that measured using a subsequent application of 10 mM caffeine.

### 3.3. The effects of a single depolarization in caffeine on s.r. Ca content

The results of Fig. 2 suggest that the cell has lost calcium after even the first pulse in the low concentration of caffeine. This will presumably lead to a decrease of s.r. Ca content and we have tested this directly by measuring the s.r. Ca content after this first pulse as shown in Fig. 4. Here, following regular stimulation, caffeine was applied to measure the s.r. Ca content. Immediately before the second exposure to 10 mM caffeine a low (200 μM) concentration of caffeine was applied for one stimulus resulting in a greatly increased systolic Ca transient. This resulted in a decrease of s.r. Ca content of 7.8 μmol l\(^{-1}\) (from 103.4 to 95.6 μmol l\(^{-1}\)). On average the s.r. Ca content was reduced from 112 ± 9 to 101 ± 8 μmol l\(^{-1}\).
Fig. 4. The effects of a single pulse in a low concentration of caffeine. (A) Original record. The traces show: top, [Ca$^{2+}$]; bottom, cumulative integral of the caffeine-evoked current. For clarity, the original current records have been omitted. Three sections of the experiment are shown: in each five stimuli are followed by the application of 10 mM caffeine to measure the s.r. Ca content. In the middle panel, 100 μM caffeine was applied immediately before (and during) the fifth pulse (*). (B) Specimen records of (from top to bottom): [Ca$^{2+}$]; membrane current; cumulative integral. The left record (o) is the fourth stimulus in the middle section of A and the right one the fifth stimulus (*).

(n = 4, P < 0.005) following a single pulse in 100 μM caffeine. These effects of the low concentration of caffeine were reversible as shown by the third determination of s.r. content. Fig. 4B shows the [Ca$^{2+}$] and current records for the control Ca transient and the one in 200 μM caffeine (*). The Na–Ca exchange current tail is enhanced by caffeine leading to a net Ca loss from the cell on this one pulse of 6.6 μmol l$^{-1}$. This predicted loss of calcium is in remarkable agreement with that measured directly from the s.r. Ca contents. On average in 4 experiments in which CICR was enhanced using 100 μM caffeine the calculated loss of calcium on the single pulse was 64 ± 11% of that measured with a subsequent application of 10 mM caffeine.

3.4. Comparison of time course of changes of s.r. Ca content and systolic [Ca$^{2+}$].

The results presented above demonstrate that enhancing Ca-induced Ca release with low concentrations of caffeine produces only a transient potentiation of the systolic Ca transient and a decrease of s.r. Ca content. Further analysis of this requires measuring changes of s.r. Ca content on a continuous beat to beat basis. In the experiment illustrated in Fig. 5 the s.r. had first been emptied by exposure to 10 mM caffeine. On subsequent stimulation there was a gradual increase of the systolic Ca transient (Fig. 5A). As shown previously [14], this is accompanied by a potentiation of the inward Ca current which gradually decays and a
Fig. 5. Calculation of sarcolemmal Ca fluxes and s.r. Ca content during s.r. refilling and application of low concentrations of caffeine. (A) Original record of [Ca^{2+}]$_i$. The cell had been exposed to 10 mM caffeine until 5 s before the start of the record. (B) Sarcolemmal Ca fluxes on each stimulus calculated from the integrals of: ▲, L-type Ca current; □, Na–Ca exchange current. (C) Net gain of Ca per pulse calculated from the difference of Ca influx and efflux. (D) Calculated gain of cell Ca.

4. Discussion

Caffeine is well known to stimulate Ca-induced Ca release by increasing the open probability of the ryanodine receptor [21]. The results in this paper show that, in agreement with previous work [11], low concentrations of caffeine produce a transient increase of the magnitude of the systolic Ca transient. In addition, this is associated with a decrease of s.r. Ca content of the order of 25%. A significant decrease in the magnitude of the s.r. Ca content can be observed after even only one stimulus in the presence of caffeine. This quantitative demonstration of a decrease of s.r. Ca content therefore extends the previous work which showed a qualitative decrease [11].

4.1. Mechanism of the changes of s.r. Ca content in low concentrations of caffeine

The present data also provide an explanation for the decrease of s.r. Ca content. We have calculated the net fluxes of calcium by integrating the entry of calcium on the L-type Ca current and subtracting the efflux on the Na–Ca exchange. In the steady state these fluxes balance. It should be noted that this method ignores Na–Ca exchange fluxes during the depolarization. This approximation has been justified previously [13]. For the present purposes, one should note that the fact that the influx and efflux balance under control conditions suggests that there is no major error.

In previous work [11] we pointed out that, in the steady-state, the Ca efflux from the cell (primarily on Na–Ca exchange) must balance the Ca influx (primarily via the Ca current). If the Ca entry into the cell is not affected then this requires that the Ca efflux is also constant. Unless the properties of the Ca efflux mechanisms are altered this requires that systolic Ca be constant. Therefore manoeuvres which affect only CICR can have
no maintained effect on systolic $[Ca^{2+}]_i$. The present paper provides direct measurements of the underlying fluxes to support this hypothesis. In the presence of the low concentration of caffeine, the stimulation of CICR increases the amount of calcium release from the s.r. and thereby increases the magnitude of the systolic Ca transient. As a consequence of this, more Ca is lost from the cell on Na–Ca exchange as demonstrated directly by the increased tail current. Experiments such as that shown in Figs. 2–4 demonstrate that this extra loss from the cell can account quantitatively for most of the decrease of s.r. Ca content measured experimentally. The calculated loss is slightly less than that measured experimentally. This may reflect the fact that there is a small transient increase of diastolic $[Ca^{2+}]_i$, (see e.g. Fig. 4) which will result in some extra Ca efflux which is not taken into account in the present calculation. The discrepancy corresponds to about 31% or approximately 6 μmol l$^{-1}$ of the measured change of s.r. Ca content (18 μmol l$^{-1}$). If we assume a cell volume of 20 pl then removal of this amount of Ca from the cell by Na–Ca exchange (assuming that 0.67 of the Ca is removed by the Na–Ca exchange) would produce a charge transfer of 8 pC. If this occurred over about 5 s then the mean current would be an undetectable 2 pA. On removal of caffeine, the decreased Ca transient decreases the loss of calcium from the cell such that there is now a net cellular gain of calcium.

### 4.2. Implications for other modulators of CICR

Since the present data show that stimulation of CICR by caffeine produces only a transient increase of systolic $[Ca^{2+}]_i$, it is worth considering other work which shows maintained effects of putative regulators of CICR. The ryanodine receptor has been shown to be modulated by phosphorylation [7] and cyclic ADP ribose [4]. Subsequent work has investigated the effects of these agents on systolic Ca and contraction in ventricular myocytes. Intracellular application of protein phosphatases produced a maintained decrease of the systolic Ca transient [6]. Similarly the application of cyclic ADP ribose produced a maintained increase of contraction [3] (although another study found no effect [22]). Furthermore an antagonist of cyclic ADP ribose produced a maintained decrease of contraction and the systolic Ca transient [2]. It has also been reported that abnormal CICR may also be responsible for the maintained reduction of the systolic Ca transient in cardiac hypertrophy and failure [10]. In our opinion, there are two possible explanations for these maintained effects. (i) Under the conditions used in those studies, the s.r. Ca content may not have decreased despite the enhanced s.r. release. This could be because the s.r. reaches a steady-state with respect to diastolic calcium. This will occur at slow rates of stimulation when the influence of the systolic fluxes will have disappeared before the next stimulus. The exact experimental conditions will determine how long it takes for this to occur. For example, in the study on the cyclic ADP Ribose antagonist [2], voltage-clamp pulses were applied at 0.3 Hz. Although this is the same as the rate used in the present work, the previous work was performed at 37°C and therefore it is likely that more equilibration will have occurred during the diastolic period. The physiologically relevant question is, of course, what happens at normal heart rates. It is likely that at typical rates, for rats and guinea-pigs of 4-8 Hz, there will not be sufficient time for equilibration to occur during diastole (even at 37°C) and therefore any stimulation of CICR will deplete the s.r. Ca content. (ii) An alternative explanation is that these conditions affect other Ca handling systems. For example it has been shown that FK-506, in addition to stimulating s.r. Ca release inhibits the Na–Ca exchange and thereby increases the filling of the s.r. [9]. Similarly, in cardiac hypertrophy it has also been suggested that there may be a reduction in s.r. Ca uptake [23,24] which would therefore be expected to decrease s.r. Ca content. It should also be noted that a recent paper has attributed the positive inotropic effect of FK-506 to a prolongation of the action potential [25]. When the experiments were performed under voltage clamp conditions, no effect on the Ca transient was observed. In that work the effects of FK-506 were studied 4 min after its application. The lack of any effect is consistent with the hypothesis of the present paper.

We consider that the above reports of maintained effects of phosphatases and cyclic ADP ribose and its antagonists cannot be produced solely by a simple effect on the ryanodine receptor. It may be that there are effects on other Ca handling pathways in the cell such as the s.r. Ca-ATPase or sarcolemmal channels and/or pumps. The results of this paper suggest that the ryanodine receptor is unlikely to be a useful target for pharmacological inotropic interventions.

We end on a teleological point. At first sight it may seem strange that mechanisms to regulate the ryanodine receptor should have evolved if they only produce transient effects. In this context it is worth considering the effects of catecholamines. These produce their inotropic effect, in part, by phosphorylating the s.r. Ca-ATPase regulatory protein phospholamban. The onset of the resulting increase of systolic $[Ca^{2+}]_i$ will be delayed by the time taken for the s.r. Ca content to increase. However, if this delayed action is combined with a transient effect due to phosphorylation of the ryanodine receptor, the net result may well be a faster increase of systolic $[Ca^{2+}]_i$. Put simply, the transient effect of stimulation of the ryanodine receptor may make the difference between escaping from the sabre-toothed tiger or not.

### Acknowledgements

This work was supported by grants from the BHF, Wellcome Trust and the European Union. M.E.D. was supported by Consejo Nacional de Investigaciones Cien-
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