Ups and downs of calcium in the heart

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Abstract

Contraction and relaxation of the heart result from cyclical changes of intracellular Ca concentration ($[Ca^{2+}]_i$). The entry of Ca^{2+} into the cell via the L-type Ca current leads to the release of more from the sarcoplasmic reticulum (SR). Compared to other regulatory mechanisms such as phosphorylation, calcium signalling is very rapid. However, since Ca^{2+} cannot be destroyed, Ca signalling can only be controlled by pumping across membranes. In the steady state, on each beat, the amount of Ca released from the SR must equal that taken back and influx and efflux across the sarcolemma must be equal. Any imbalance in these fluxes will result in a change of SR Ca content and this provides a mechanism for regulation of SR Ca content. These flux balance considerations also explain why simply potentiating Ca release from the SR has no maintained effect on the amplitude of the Ca transient. A low diastolic $[Ca^{2+}]_i$ is essential for cardiac relaxation but the factors that control diastolic $[Ca^{2+}]_i$ are poorly understood. Recent work suggests that flux balance is also important here. In particular, decreasing SR function decreases the amplitude of the systolic Ca transient and the resulting decrease of Ca efflux results in an increase of diastolic $[Ca^{2+}]_i$ to maintain total efflux.

Introduction

It is well-established that contraction of the heart is controlled by changes of cytoplasmic calcium concentration ($[Ca^{2+}]_i$). $[Ca^{2+}]_i$ must be high enough in systole to activate the contractile proteins in order to pump blood out of the heart. Equally importantly, it must fall during diastole to low enough levels that the muscle of the heart relaxes so that the chambers can refill with blood. The aim of this article is to discuss factors responsible for both the rise and fall of $[Ca^{2+}]_i$.

The importance of calcium in contraction of the heart was originally demonstrated by Sydney Ringer (Ringer, 1883). I have recently described both the serendipitous nature of his discovery and the

manner in which it was published (Eisner, 2014). In the subsequent 130 years it has become clear that changes of intracellular calcium concentration ($[Ca^{2+}]_i$) regulate the function of virtually all organs. It took, however, almost one hundred years after Ringer's discovery to measure directly the transient elevation of $[Ca^{2+}]_i$ (the calcium transient) which underlies cardiac contraction (Allen & Blinks, 1978; Allen & Kurihara, 1980).

Calcium versus other signalling modalities. Calcium has its actions by binding to proteins and thereby changing their shape and properties (Fig. 1A). In the case of striated muscle, calcium binds to troponin and the resulting movement of tropomyosin allows actin and myosin to interact. There are, of course, many other ways to alter the properties of proteins including phosphorylation and nitrosylation. This immediately raises the question of what are the advantages and disadvantages of using calcium as opposed to other ways of modifying protein structure? One great advantage of calcium is its speed of action. $[Ca^{2+}]_i$ rises and Ca^{2+} ions bind to troponin very quickly and. In cardiac muscle contraction can be 50% complete within 200 ms (Fig 1A). In contrast regulation by phosphorylation is much slower. Fig 1B shows the effects of beta adrenergic stimulation on the Ca current. This takes about 20 s to be 50% complete (Frace *et al.*, 1993). This slower timescale is due to the many steps involved; agonist binding to the receptor, generation of cyclic AMP, and, finally, the activation of protein kinase A which then phosphorylates the L-type channel. Although signalling by phosphorylation is slower than that by Ca^{2+}, it has the advantage that it can be regulated in a much more subtle way. Specific phosphodiesterases can break down cAMP and various regulators affect the activities of the kinases and phosphatases. Another significant advantage of control by phosphorylation is that it can be reversed simply by dephoshorylation. In contrast, Ca^{2+} ions cannot be destroyed. The only way to reverse Ca^{2+} binding is to pump the Ca^{2+} across a membrane either out of the cell or into an intracellular store. Put simply, Ca^{2+} must be recycled and many of the results and conclusions of the remainder of this review follow from this.

The above argument needs some qualification. Although it describes well the situation in striated muscle, where Ca^{2+} binding is followed rapidly by contraction, in other tissues the steps subsequent to Ca^{2+} binding may involve slower, enzymatic processes. This is for example the case in smooth muscle where Ca^{2+} binds to calmodulin, activating the enzyme myosin light chain kinase which, in turn, phosphorylates myosin. There is therefore a longer delay between the rise of $[Ca^{2+}]_i$ and that of contraction (Shabir *et al.*, 2004). It should also be noted that factors other than speed are also involved in the evolution of calcium signalling (Case *et al.*, 2007).

Flux balance. The events underlying Ca cycling during the heartbeat are illustrated in Fig 2. (See Bers (2001), Bers (2008) ,Eisner *et al.* (2017) for reviews). Ca^{2+} enters the cell through the L-type Ca^{2+} channel resulting in a large increase of $[Ca^{2+}]_i$ in the small dyadic space between the surface membrane/transverse tubule and the sarcoplasmic reticulum (SR). This Ca^{2+} binds to the SR Ca release channel (Ryanodine Receptor, RyR) making it open and releasing a much greater amount of Ca^{2+} into the cytoplasm from the SR, a process known as calcium-induced calcium release (Fabiato, 1985). Relaxation requires that $[Ca^{2+}]_i$ be lowered to resting levels. This occurs in two ways. (1) Ca^{2+} is pumped back into the SR by SERCA, the sarco/endoplasmic reticulum Ca^{2+}-ATPase. The activity of SERCA is regulated, in part by $[Ca^{2+}]_i$ and also by the inhibitory accessory protein, phospholamban. Phosphorylation of phospholamban, as occurs during beta-adrenergic stimulation relieves the inhibition and accelerates SERCA (Kirchberber *et al.*, 1975). (2) Ca is also removed from the cell, largely by sodium calcium exchange (NCX) which uses the entry of three Na^+ to power the exit of one Ca^{2+} from the cell (Kimura *et al.*, 1986). The resulting electrogenic current can be used to measure the activity of NCX in the intact cell. It is important to note that the various calcium fluxes must be balanced on each cycle. In other words, in the steady state, the calcium influx into the cell must exactly equal that pumped out. Likewise, the amount of Ca^{2+} released from the SR must equal

that pumped back. This calcium flux balance is an example of the need to recycle calcium mentioned above.

Control of the amplitude of the calcium transient

The size of the systolic Ca transient is not fixed. It increases, for example, during exercise due to β-adrenergic stimulation (Endoh & Blinks, 1988) and decreases in heart failure (Beuckelmann *et al.*, 1992). Three of the steps indicated in Fig. 2A are potential control points. (i) The size of the L-type Ca^{2+} current; the larger the current, the greater the triggering of RyRs to open. (ii) The ease with which the RyRs open. I will show later that this is probably not an important control point. (iii) The amount of Ca in the SR. Fig 2B shows that, as SR Ca increases, there is a steep increase of the amplitude of the Ca^{2+} transient (Bassani *et al.*, 1995). On average, the amplitude of the Ca^{2+} transient is proportional to the cube of SR Ca content (Trafford *et al.*, 2000). Several factors account for the steepness. An increase of SR luminal Ca increases the driving force for Ca^{2+} to leave the SR. It will also increase the open probability of the RyR. This can occur either by Ca^{2+} binding directly to sites on the luminal part of the RyR or by the Ca^{2+} which flows out through the RyR binding to sites on the cytoplasmic surface (Guo *et al.*, 2012). Whatever the origin of the steep relationship, it is of great significance. For example, a small reduction of SR Ca content in cardiac hypertrophy will have a much larger fractional effect on the amplitude of the systolic Ca transient (Díaz *et al.*, 2004). Furthermore, the steep dependence means that regulation of the amplitude of the systolic Ca transient requires exquisitely precise control of SR Ca content. This therefore raises the question of how SR Ca content is controlled.

Control of SR Ca content

Much work has shown that SR Ca content in the heart is controlled by a simple, negative feedback mechanism (Trafford *et al.*, 1997). This depends on the fact that (i) the Ca^{2+} transient amplitude is a steep function of SR Ca content and (ii) fluxes of Ca^{2+} across the sarcolemma are sensitive to the amplitude of the Ca transient. An example of this mechanism is shown in Fig 3A. A high (10 mM) concentration of caffeine had been applied to empty the SR. Following removal of caffeine, the cell was electrically stimulated. The Ca transient was initially very small as a result of the low SR Ca content but then increased over the course of about ten beats as the SR refilled. The underlying changes of sarcolemmal fluxes are illustrated in Fig. 3B. When the Ca transient is small (a), there is less Ca^{2+}-dependent inactivation of the L-type current (Sipido *et al.*, 1995) and therefore increased influx. There is also less efflux on NCX. The cell is therefore not in Ca flux balance and gains calcium. The resulting increase of SR Ca content increases the amplitude of the Ca^{2+} transient making the L-type current inactivate more quickly, decreasing Ca^{2+} influx and there is also more efflux on NCX such that a new steady state is reached.

These effects of the Ca^{2+} transient on sarcolemmal Ca^{2+} fluxes underpin a simple yet powerful negative feedback loop which controls SR Ca content (Fig. 3C). This can be illustrated by considering the consequence of an increase of SR Ca content. This will increase the amplitude of the Ca^{2+} transient which, in turn, will increase Ca^{2+} efflux and decrease influx, thereby decreasing cell and SR Ca content. Not only does this mechanism regulate SR Ca content but it is fundamental to ensuring that the fluxes of Ca^{2+} into and out of the cell and across the SR membrane are balanced on each beat. Any imbalance of Ca^{2+} flux across the surface membrane will result in a change of cell and SR Ca leading to an alteration of the amplitude of the Ca^{2+} transient and, thence to changes of Ca^{2+} fluxes that will return the cell to Ca^{2+} flux balance. Ca^{2+} fluxes can, of course, be out of balance but only for short periods. One example has already been shown; the effect of stimulating when the SR is empty. Under these conditions, the cell is initially not in Ca^{2+} flux balance; influx is greater than

efflux until the SR Ca content and Ca^{2+} efflux increase sufficiently that efflux equals influx and the cell is back in flux balance. Another, well-known example is when the frequency of stimulation is changed or stimulation is recommenced after a pause. For example, in species other than rodents, when stimulation is stopped Ca^{2+} leaks out of the SR (Bridge, 1986). The SR refills during stimulation resulting in a gradual increase of the amplitude of the Ca transient and contraction (Allen *et al.*, 1976).

The effects of increasing RyR opening. A particularly striking demonstration of the consequences of the maintenance of calcium flux balance is provided by the effects of increasing the open probability of the RyR. Fig 4 demonstrates that the application of 500 μM caffeine to increase RyR opening produces an immediate increase of the amplitude of the systolic Ca transient. This increase is, however, short-lived and the amplitude of the Ca^{2+} transient decays to basal levels within a few beats (Trafford *et al.*, 2000). The explanation of this result is as follows. Under control conditions, Ca^{2+} efflux and influx are equal. The increase in the size of the systolic Ca^{2+} transient produced by caffeine increases Ca^{2+} efflux until it is greater than influx resulting in a decrease of SR Ca content. (See Shannon *et al.* (2005) for detailed mathematical modelling of this phenomenon). The decrease of SR Ca content in Fig. 4 was calculated from the change of NCX flux. More direct evidence for it comes from showing that the amount of SR Ca that can be released by a high concentration (10 mM) of caffeine is also decreased (Trafford *et al.*, 1998) and measuring the decrease of SR Ca directly with a low affinity Ca indicator (Greensmith *et al.*, 2014). The decrease of SR Ca, in turn, decrease the amplitude of the Ca transient until a new steady state is reached at which Ca^{2+} influx and efflux are again equal. Similar transient effects are seen for other manoeuvres which affect the opening of the RyR (Choi *et al.*, 2000); see Eisner *et al.* (2009) for review. This transient response of Ca^{2+} transient amplitude to an increase of RyR opening can be thought of as an emergent property of

the system. It results because the various, independent Ca transporters are effectively coupled by their dependence on cytoplasmic and SR [Ca^{2+}].

The regulation of resting and diastolic $[Ca^{2+}]_i$

For the heart to work properly as a pump, diastolic $[Ca^{2+}]_i$ must be sufficiently low that the ventricle is relaxed and can therefore refill with blood between beats. An increase of diastolic force, accompanied by impaired relaxation is seen in many patients with heart failure. Furthermore, heart failure with preserved ejection fraction (HFpEF) is increasingly being regarded as an important clinical problem (Borlaug & Paulus, 2011). Such patients have symptoms of heart failure but normal ejection fractions and their major clinical problem is diastolic. While other factors may well also contribute to diastolic dysfunction, there is evidence that diastolic $[Ca^{2+}]_i$ is increased in patients with heart failure (Gwathmey *et al.*, 1987; Gwathmey *et al.*, 1991; Beuckelmann *et al.*, 1992; Sipido *et al.*, 1998; Runte *et al.*, 2017). It is therefore important to understand how diastolic $[Ca^{2+}]_i$ is regulated.

Control of resting $[Ca^{2+}]_i$. Many studies have investigated the regulation of $[Ca^{2+}]_i$ in unstimulated (resting) cardiac preparations. Early work, performed before the introduction of Ca^{2+}-sensitive indicators, used resting force as a bio-assay of $[Ca^{2+}]_i$. The role of the NCX, for example, was originally demonstrated by examining the effects of changing extracellular sodium concentration (Niedergerke, 1963; Chapman & Tunstall, 1980). Subsequent work measuring intracellular Na concentration showed that the voltage-dependent NCX played a major role in controlling resting force and therefore $[Ca^{2+}]_i$ (Eisner *et al.*, 1983). The advent of fluorescent Ca^{2+} indicators allowed more direct measurements and also identified a role for the plasma membrane Ca-ATPase in the regulation of resting $[Ca^{2+}]_i$ (Lamont & Eisner, 1996). What is less well-established is the route by which Ca^{2+} enters a resting cell. One possibility might be the random opening of L-type Ca^{2+} channels but the likely flux would be low at the normal resting potential where the open probability is low.

Work on rat ventricular myocytes found evidence for three voltage-sensitive mechanisms involved in the control of resting $[Ca^{2+}]_i$. (i) NCX; the greater the depolarization, the more the Ca^{2+} influx. (ii) L-type Ca^{2+} current; depolarization to about zero mV increased influx but the magnitude decreased on further depolarization as the driving force for Ca^{2+} entry was reduced. (iii) Finally, there was an increase of $[Ca^{2+}]_i$ on hyperpolarization, attributed to increased driving force (Kupittayanant *et al.*, 2006) on an un-gated Ca^{2+} entry pathway. Gadolinium at a concentration of 250 μM was the only substance found to completely inhibit this Ca entry leaving its identity unresolved. One of the classes of channels which are sensitive to gadolinium is the so called transient receptor potential (TRP). There has been considerable interest in the properties of such channels in the heart with them being implicated in the development of hypertrophy (Gao *et al.*, 2012) and the cell damage in muscular dystrophy (Lorin *et al.*, 2015). Another channel which may contribute to the background Ca entry mechanism is that responsible for store-operated Ca entry since its inhibition has been reported to decrease resting $[Ca^{2+}]_i$ in HL-1 cells (Touchberry *et al.*, 2011). The importance of store-operated channels in normal adult cardiac myocytes is, however, still controversial (see Eisner *et al.* (2017) for review).

Diastolic $[Ca^{2+}]_i$. At first sight one might think that the diastolic level of resting $[Ca^{2+}]_i$ would be similar to the resting level in an unstimulated cell. In point of fact this is not the case and diastolic is higher (Fig 5). This is because, in the steady state, in a resting cell, $[Ca^{2+}]_i$ is controlled solely by the surface membrane with the sarcolemmal Ca influx being exactly balanced by efflux (Eisner *et al.*, 1984; Friel & Tsien, 1992; Rios, 2010). The question then is what does control diastolic $[Ca^{2+}]_i$? In recent work we have investigated the effects of interfering with SR function. This work was prompted by the fact that heart failure affects the SR by both making the RyR leaky and decreasing SERCA activity; see Bers (2014) for review.

The experiment illustrated in Fig 5A shows the effects on diastolic $[Ca^{2+}]_i$ of making the RyR leaky with caffeine (1 mM) (Sankaranarayanan *et al.*, 2017). This is a higher concentration than was used

in Fig. 4 and results in a release of Ca^{2+} from the SR even in the absence of stimulation indicating a marked decrease of SR Ca. The grey trace shows that, under basal conditions, the diastolic $[Ca^{2+}]_i$ at 2 Hz stimulation is only slightly greater than resting $[Ca^{2+}]_i$. The subsequent application of caffeine decreased systolic $[Ca^{2+}]_i$ and increased diastolic $[Ca^{2+}]_i$. After removing caffeine, application of the β-adrenergic agonist isoprenaline (ISO) increased systolic $[Ca^{2+}]_i$. The subsequent addition of caffeine again decreased systolic $[Ca^{2+}]_i$ and produced a more marked increase of diastolic $[Ca^{2+}]_i$ than was seen in the absence of ISO. The average data (Fig. 5B) confirm the effects of caffeine on diastolic and systolic $[Ca^{2+}]_i$. It is also noteworthy that, in the absence of caffeine, ISO produces a large increase of the amplitude of the Ca transient with little or no effect on diastolic $[Ca^{2+}]_i$. In contrast, in the presence of caffeine, the effect of ISO on the amplitude is greatly diminished and that on diastolic is increased.

The maintained decrease of systolic $[Ca^{2+}]_i$ contrasts with the transient potentiation with no steady state effect seen in Fig. 4. The explanation is that the higher concentration of caffeine results in a large release of calcium from the SR thereby greatly decreasing SR Ca. If SR Ca falls sufficiently then, even if RyR opening is potentiated enormously and all the Ca^{2+} is released from the SR, the Ca^{2+} transient will be smaller than in control (Negretti *et al.*, 1993; Sankaranarayanan *et al.*, 2016). A similar argument has been used to explain why RyR potentiation decreases the amplitude of the Ca^{2+} transient in heart failure (Belevych *et al.*, 2007). An explanation of the associated increase of diastolic $[Ca^{2+}]_i$ is that it is a direct result of the decrease of systolic $[Ca^{2+}]_i$ and the need to preserve flux balance. Before caffeine is added, influx and efflux will be equal. In the presence of caffeine the smaller Ca transient will decrease the systolic Ca^{2+} efflux to less than the influx leading to the cell gaining Ca. This is compensated by the increase of diastolic $[Ca^{2+}]_i$ which will increase efflux. Assuming that Ca^{2+} influx is unaffected by caffeine then the total (systolic plus diastolic) efflux must be the same in the absence and presence of caffeine. If we assume that NCX activity is proportional to $[Ca^{2+}]_i$ (Barcenas-Ruiz *et al.*, 1987) then one would expect the time-averaged $[Ca^{2+}]_i$ concentration

to be constant. That this is indeed the case is demonstrated by the low-pass filtered trace of Fig 5A and the mean data of Fig. 5B which show that average $[Ca^{2+}]_i$ is unaffected by making the RyR leaky with caffeine (Sankaranarayanan *et al.*, 2017). Fig 6 demonstrates the results of stimulating over a wider range of frequencies. The average level of $[Ca^{2+}]_i$ is unaffected by addition of caffeine (Fig 6A – see Fig 6B for mean data). Increasing frequency increases average $[Ca^{2+}]_i$ but in a manner that tends to saturation. The average level of $[Ca^{2+}]_i$ can usefully be compared with the calcium influx into the cell. As the frequency of stimulation is increased, the influx per pulse decreases due to increased inactivation (Fig. 6C) (Sipido *et al.*, 1998; Dibb *et al.*, 2007). The influx per unit time, however, increases in a saturating manner with frequency. The linear relationships between influx per unit time and average $[Ca^{2+}]_i$ are shown in Fig. 6D. The lines are statistically identical in the absence and presence of caffeine. One noteworthy feature of Fig 6D is that, even when there is no Ca^{2+} influx, there is still a finite level of $[Ca^{2+}]_i$. The extrapolation of the fitted lines provides horizontal intercepts of the order of 4 $\mu mol.l^{-1}.s^{-1}$ suggesting that, in addition to the L-type Ca^{2+} current, there is a continuous influx of Ca^{2+} into the cell of this magnitude. This is a similar magnitude to the value obtained with a different method referred to above. It therefore appears that, at least under the conditions of these experiments, Ca^{2+} influx consists of two components: (i) background and (ii) L-type Ca^{2+} current. There will doubtless be other routes including possible entry through reverse mode NCX but these are not resolved here. The tight correlation between Ca^{2+} entry and average $[Ca^{2+}]_i$ demonstrates that it is the influx per unit time which sets the average level of $[Ca^{2+}]_i$; the properties of the SR simply determine the relative contributions of diastolic and systolic $[Ca^{2+}]_i$ to the average. A good example of this is the effect of β-adrenergic stimulation on $[Ca^{2+}]_i$. Under normal conditions (Fig 5B) this results in an increase of systolic $[Ca^{2+}]_i$ with little or no effect on diastolic. In contrast, when the RyR is made leaky with ryanodine, the bulk of the increase is diastolic.

In addition to diastolic and systolic $[Ca^{2+}]_i$, calcium waves also contribute to Ca^{2+} efflux and average $[Ca^{2+}]_i$. An increase of SR Ca content increases the frequency of Ca sparks resulting in the occurrence of waves of calcium induced calcium release which propagate through the cell (Cheng *et al.*, 1996). Some of the calcium in these waves is pumped out of the cell on NCX and the resulting current has been shown to produce delayed afterdepolarizations and thereby initiate various arrhythmias (Ferrier *et al.*, 1973; Mechmann & Pott, 1986). Despite their arrhythmogenic nature, these Ca waves may serve a useful function in providing a route for Ca efflux, in addition to elevated diastolic $[Ca^{2+}]_i$ under conditions of calcium overload (Díaz *et al.*, 1997). More generally, at a given Ca^{2+} influx, the sum of the effluxes produced by the three components of efflux; systolic, diastolic, and Ca waves must be constant. One example of the consequences of this is shown in Fig. 7. Under basal conditions (left hand panel), the systolic efflux (SE) balances Ca^{2+} influx. The application of a high concentration of isoprenaline (1μM, middle panels), increases Ca^{2+} influx on the L-type current (I) and results in Ca^{2+} waves. It is largely the Ca^{2+} efflux associated with the wave (DE) which balances the increased influx due to isoprenaline. Finally (right hand panel), tetracaine, which decreases RyR opening, abolishes the waves and the associated efflux. Ca^{2+} flux balance is now maintained by an increase of systolic efflux due to the increased Ca^{2+} transient (Venetucci *et al.*, 2006).

The previous section has investigated the effects of disabling SR function by making the RyR leaky. We have also studied the effects of decreasing Ca^{2+} uptake into the SR by decreasing SERCA activity with thapsigargin. As has been shown previously (Janczewski & Lakatta, 1993; Negretti *et al.*, 1993), the resulting decrease of SR Ca content decreases the amplitude of the Ca transient, Fig 8 also shows that there is an increase of diastolic $[Ca^{2+}]_i$. This, in turn, will increase the diastolic efflux thereby compensating for the decrease of systolic efflux. Therefore, no matter whether the amplitude of the Ca transient is decreased by making the RyR leaky or decreasing SERCA activity, the result is a compensatory increase of diastolic $[Ca^{2+}]_i$. Fig 8B shows, again, that increasing stimulation

frequency increases average $[Ca^{2+}]_i$. Thapsigargin has a much greater effect on diastolic $[Ca^{2+}]_i$ at the higher stimulation rate. This is presumably because, at lower rates, there is time for $[Ca^{2+}]_i$ to fall to close to the resting (unstimulated) level. Indeed, as shown in Fig 8A, in the absence of stimulation thapsigargin has no effect on resting $[Ca^{2+}]_i$.

From these experiments, we therefore conclude that systolic $[Ca^{2+}]_i$ is a major factor regulating diastolic $[Ca^{2+}]_i$. The overall regulation of diastolic $[Ca^{2+}]_i$ is yet another example of the importance of considering Ca flux balance. A given Ca^{2+} influx must be balanced by an equal efflux. Assuming that the properties of NCX are constant and the rate of NCX pumping is proportional to $[Ca^{2+}]_i$, then the level of Ca influx sets the average level of $[Ca^{2+}]_i$. The relative diastolic and systolic levels that determine the average are determined by factors such as SR function. Making the RyR leaky or inhibiting SERCA will result in a situation with elevated diastolic and decreased systolic $[Ca^{2+}]_i$ compared to the situation with a normal SR. All other things being equal, a decrease of systolic $[Ca^{2+}]_i$ as occurs in heart failure would be expected to be associated with elevated diastolic $[Ca^{2+}]_i$. What actually happens will depend, also, on the Ca^{2+} influx. Although many studies in heart failure find that the L-type current is unaffected, some work has reported a decrease (see Benitah *et al.* (2010) for review). The latter case should be associated with a decrease of average $[Ca^{2+}]_i$ thereby mitigating the expected rise of diastolic $[Ca^{2+}]_i$. It should, however, also be noted that the situation is potentially more complex. Firstly, the decrease of the Ca transient may slow calcium-dependent inactivation of the L-type current and thereby maintain or even increase Ca^{2+} influx. Secondly, the prolongation of the action potential which occurs in heart failure will increase Ca^{2+} entry through the L-type current.

Conclusions

I have referred extensively to the fact that the ventricular myocyte is in calcium flux balance: on each beat, in the steady state, the amount of Ca^{2+} that enters the cell equals that which leaves. As I have reviewed, this has important consequences for cellular calcium cycling and cardiac function. It underlies the regulation of SR Ca content. It explains why an increase in RyR open probability has no effect, in the steady state, on the amplitude of the Ca transient. Finally, it explains the inverse relationship between the levels of diastolic and systolic $[Ca^{2+}]_i$ as well as the importance of average $[Ca^{2+}]_i$.

Abstract figure. Flow diagram showing Ca^{2+} flux balance illustrating the response to a situation in which Ca^{2+} influx exceeds efflux. With normal SR function this will increase SR Ca content and increase systolic $[Ca^{2+}]_i$. If SR function is impaired there will be an increase of diastolic $[Ca^{2+}]_i$. In both cases this will result in an increase of Ca^{2+} efflux such that efflux equals influx.

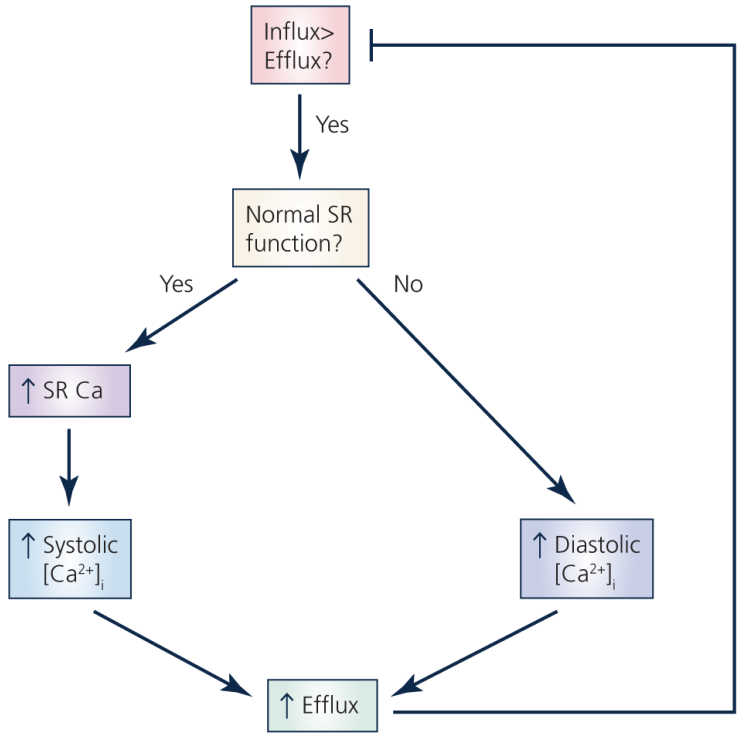

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Figure 1. Comparison of control by calcium and phosphorylation. **A**. Calcium. The upper part shows a schematic diagram of a protein changing shape when it binds Ca^{2+}. The lower part shows timecourse of (from top to bottom): action potential, $[Ca^{2+}]_i$, and cell shortening recorded from a sheep ventricular myocyte. (Record kindly provided by K.M. Dibb). **B**. Phosphorylation. The upper part shows the change of shape produced by phosphorylation and its reversal by dephosphorylaton with a phosphatase. The steps between binding to the receptor and phosphorylation are shown: agonist binds to the β-adrenergic receptor (β-AR) which, via a G-protein, activated adenylate cyclase (AC) producing cyclic AMP (cAMP) which activates protein kinase A (PKA). The lower part shows the timecourse of the effects of isoprenaline (ISO) on the L-type Ca^{2+} current in a frog ventricular myocyte. (Reproduced with permission from Frace *et al.* (1993)). Cartoons kindly drawn by Jessica Caldwell.

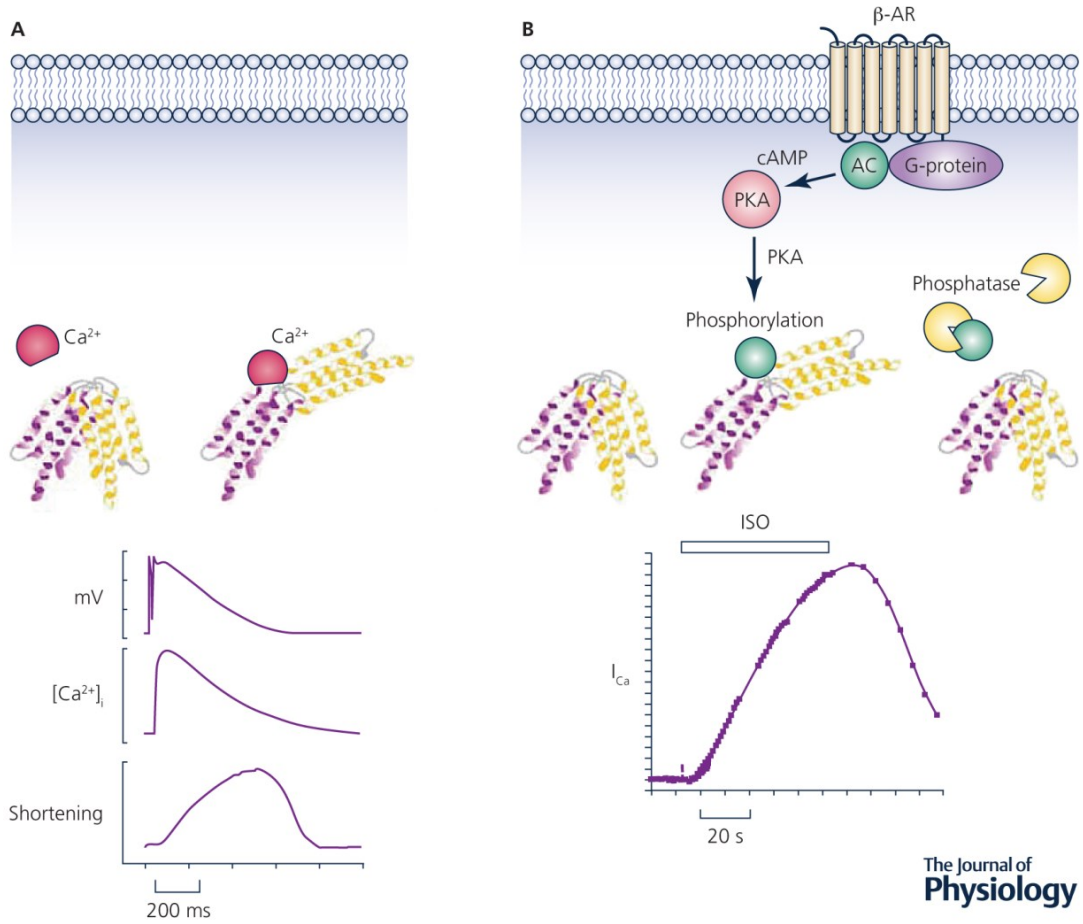


Figure 2. **Control points in cellular calcium cycling**. A, schematic diagram showing Ca^{2+} fluxes. (1) Ca^{2+} enters the cell via the L-type Ca current. (2) Ca^{2+} is released via the RyR from the SR. (The amplitude of the Ca transient depends, therefore, on the sum of Ca^{2+} entry and release from the SR.) (3) Ca^{2+} is pumped back into the SR by SERCA and pumped out of the cell largely via NCX. B. The dependence of the amplitude of the Ca transient on SR Ca content. Redrawn from Trafford *et al.* (1997).

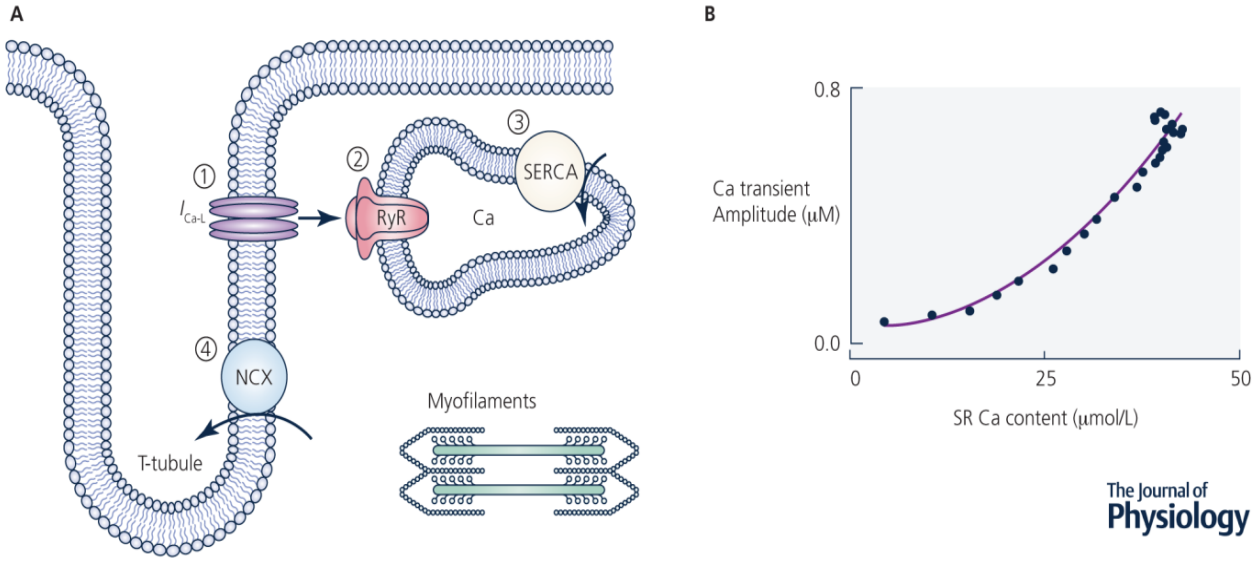


Figure 3. Negative feedback control of SR Ca content. A. Timecourse. The trace shows $[Ca^{2+}]_i$ from a ferret ventricular myocyte stimulated with voltage-clamp pulses. Before the record began, the cell had been exposed (in the absence of stimulation) to caffeine (10 mM) to empty the SR. Caffeine was then removed. The trace shows the effects of recommencing stimulation. B. Expanded traces of L-type current (left) and NCX (right). In both panels the traces correspond to the points in A; i.e. a is the first record after commencing stimulation and b the steady state. (Traces in A and B redrawn from Trafford *et al.* (1997)). C. Schematic diagram of negative feedback loop. An increase of SR Ca increases the amplitude of the Ca^{2+} transient. This decreases Ca^{2+} influx via the L-

type Ca^{2+} current (1) and increases efflux on NCX (2) leading to a decrease of cell and therefore SR Ca content. A and B redrawn from Trafford *et al.* (1997).

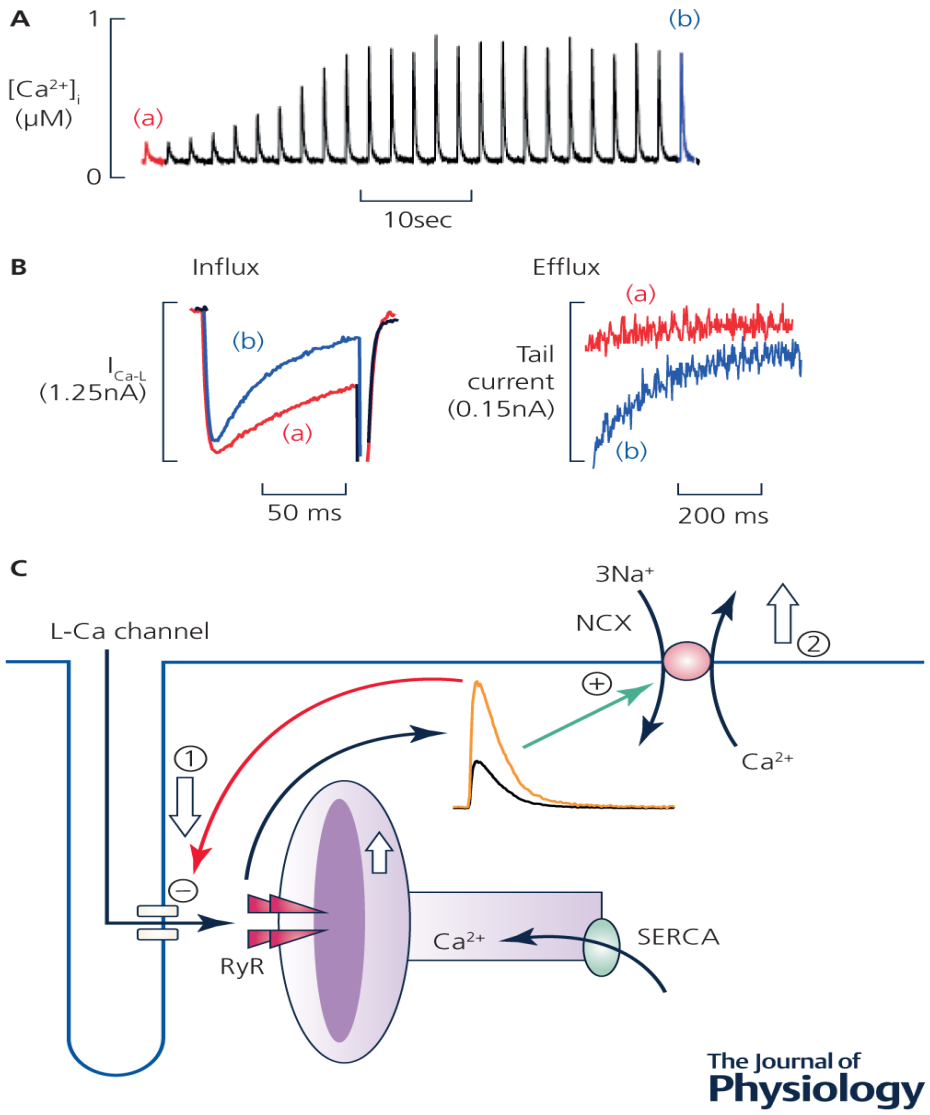


Figure 4. The effects of potentiating RyR opening. The effects of adding caffeine (500 μM) to increase RyR opening in a rat ventricular myocyte. Traces show (from top to bottom): $[Ca^{2+}]_i$;

sarcolemmal Ca^{2+} fluxes (influx on L-type Ca^{2+} current, efflux on NCX; predicted change of SR Ca content. Redrawn from Trafford *et al.* (2000)

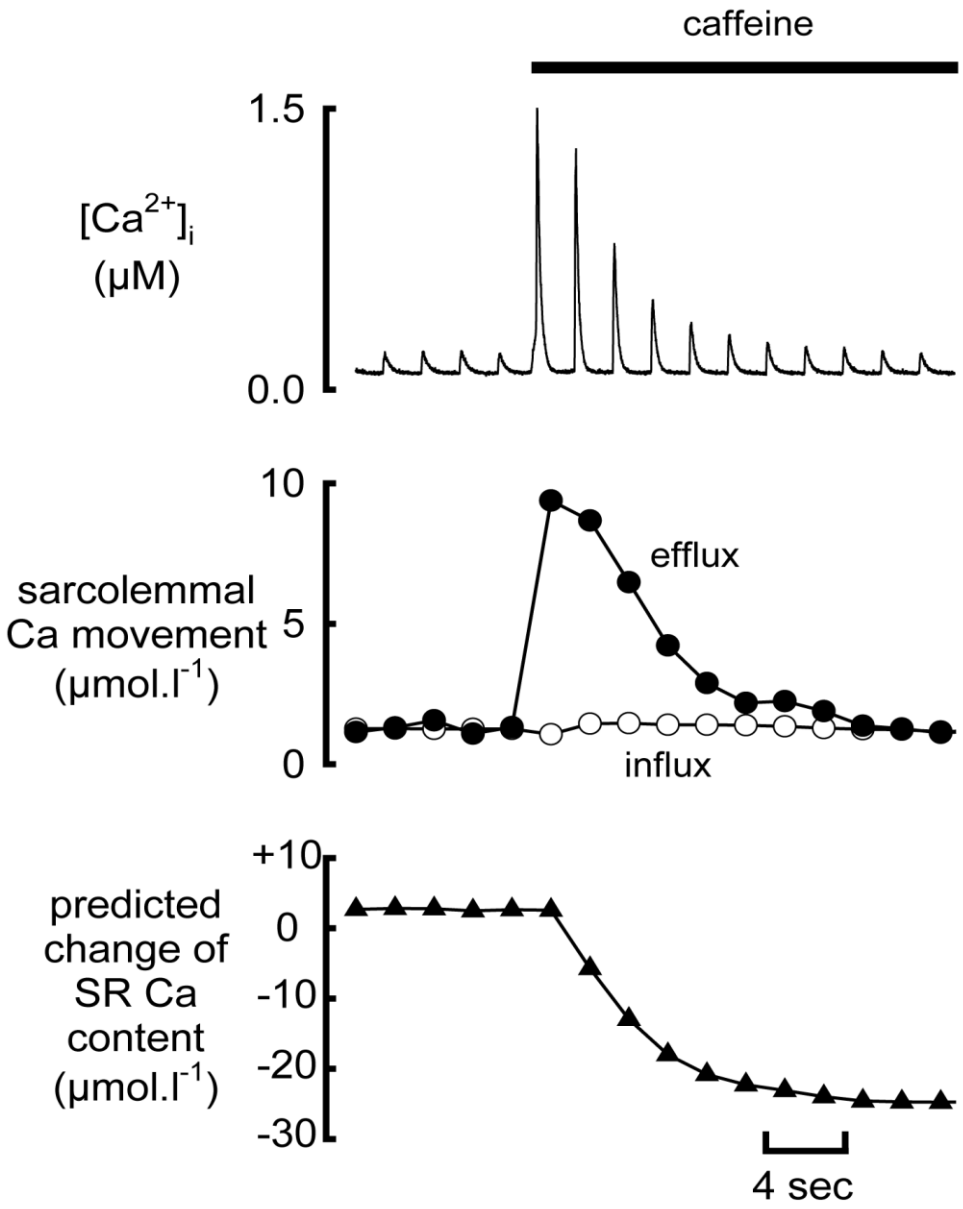


Figure 5. Effects of caffeine and isoprenaline on diastolic and systolic $[Ca^{2+}]_i$ **.** A. Timecourse (rat ventricular myocyte). The grey trace shows $[Ca^{2+}]_i$ during rest and stimulation at 2 Hz. The blue is a low-pass filtered version. Caffeine (Caf, 1 mM) and isoprenaline (ISO, 1 μM) were applied as shown. B. Mean data showing normalized values for the amplitude of the Ca^{2+} transient, diastolic and

average $[Ca^{2+}]_i$. In each group the unlabelled bar is control; C, caffeine; I, ISO; I+C, ISO + caffeine. Reproduced from Sankaranarayanan *et al.* (2017)

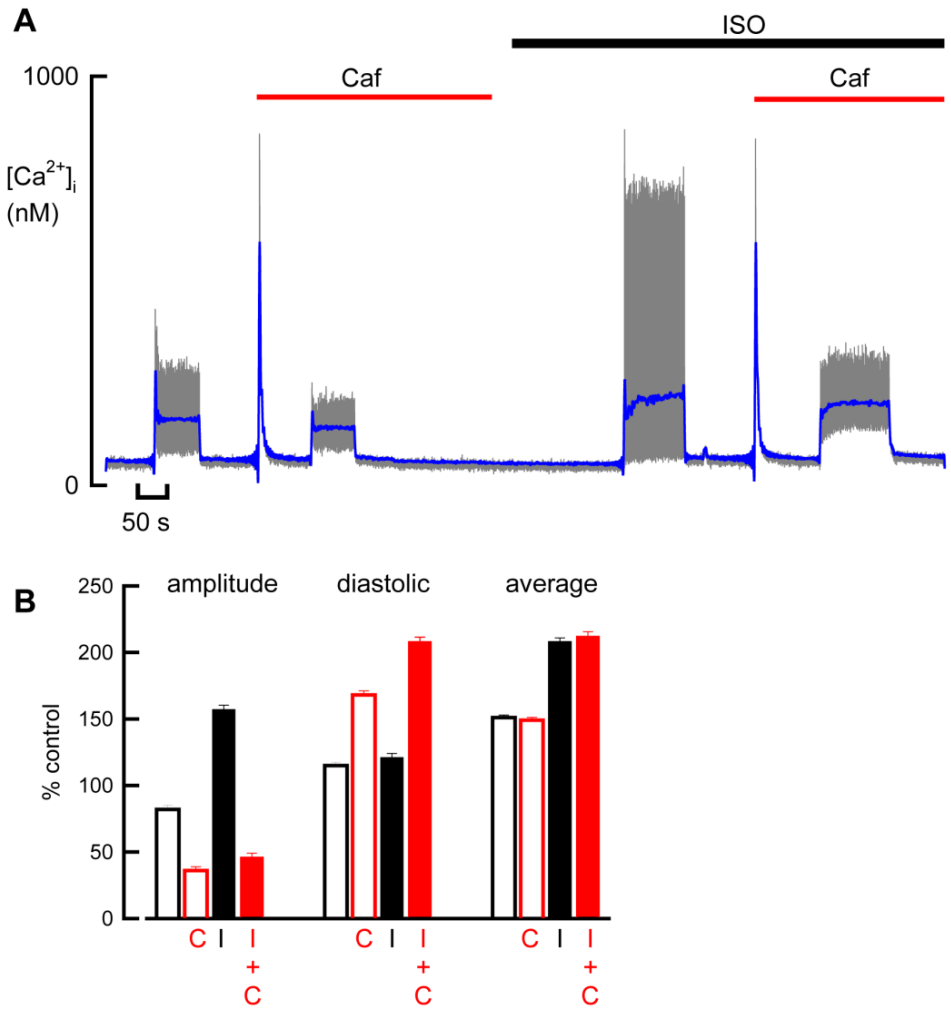


Figure 6. Interactions between RyR leak and stimulation frequency on diastolic, systolic and average $[Ca^{2+}]_i$. A, timecourse (rat ventricular myocyte). Stimulation frequency was altered and 1 mM caffeine (Caf) applied as shown above. B, mean data showing (from top to bottom): amplitude, diastolic, average $[Ca^{2+}]_i$, Ca^{2+} influx per pulse, Ca^{2+} influx per second. C, top, specimen Ca currents; bottom, integrated Ca^{2+} influx at the frequencies indicated. D, average $[Ca^{2+}]_i$ as a function of Ca^{2+}

influx per second. In all panels the red symbols indicate the presence of caffeine. Reproduced from Sankaranarayanan *et al.* (2017).

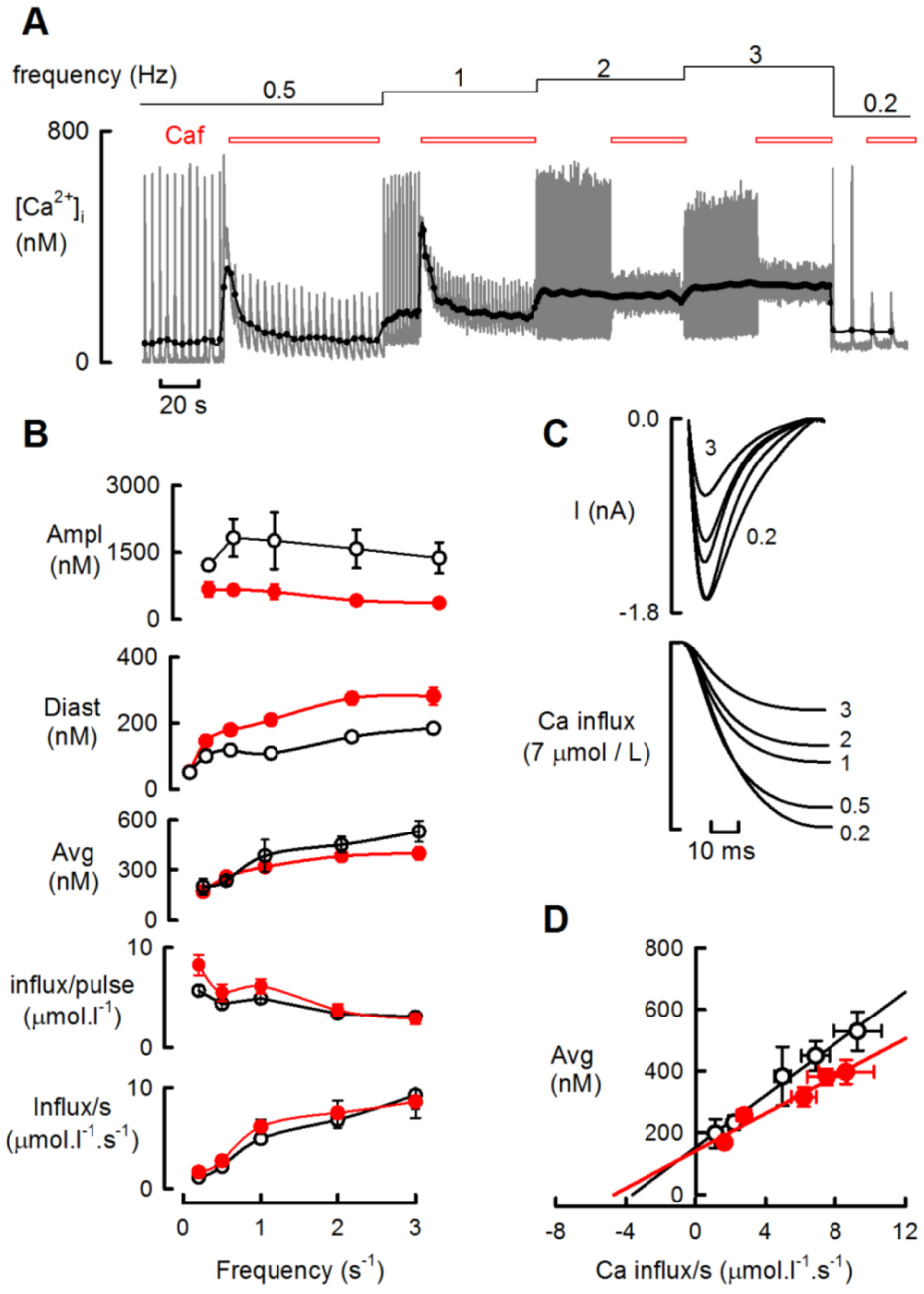


Figure 7. Ca efflux associated with Ca waves. In each panel traces show (from top to bottom): $[Ca^{2+}]_i$; membrane current (both low gain and amplified); Ca^{2+} balance (the calculated change of total intracellular Ca concentration). Panels show (from left to right): control; addition of isoprenaline (ISO,1 μM); addition of isoprenaline (1μM) and tetracaine (50 μM). Experiment performed on a rat ventricular myocyte. Reproduced from Venetucci *et al.* (2006).

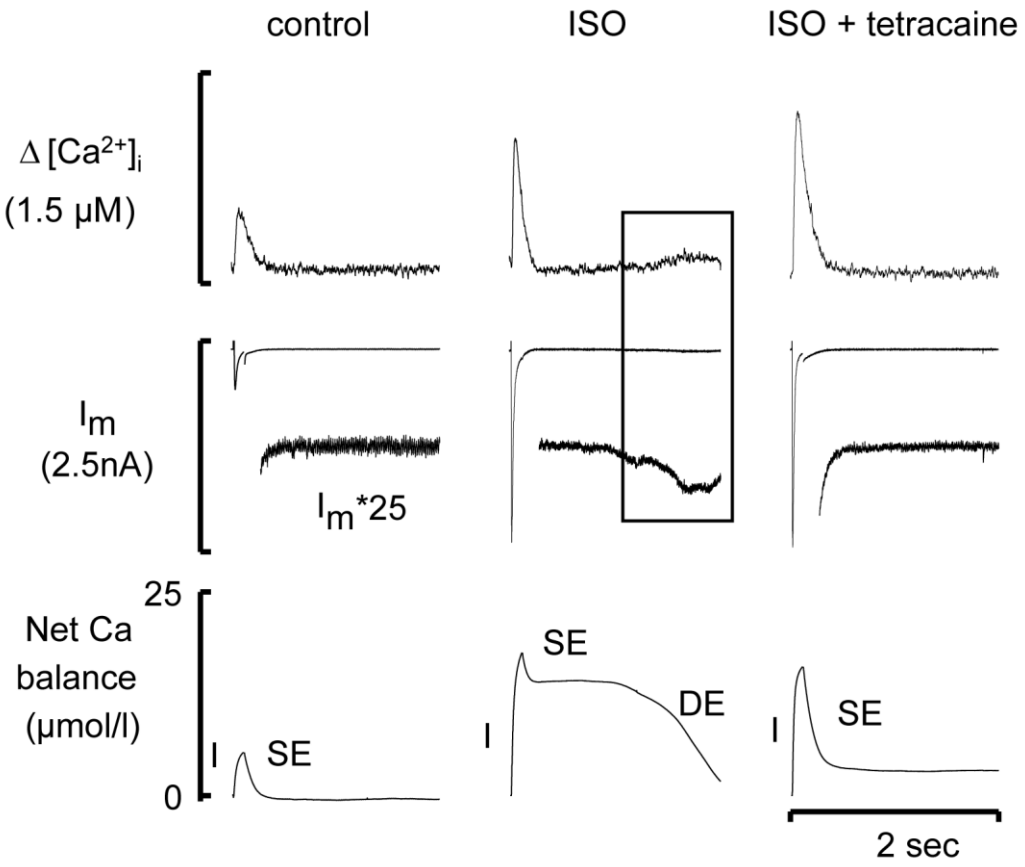
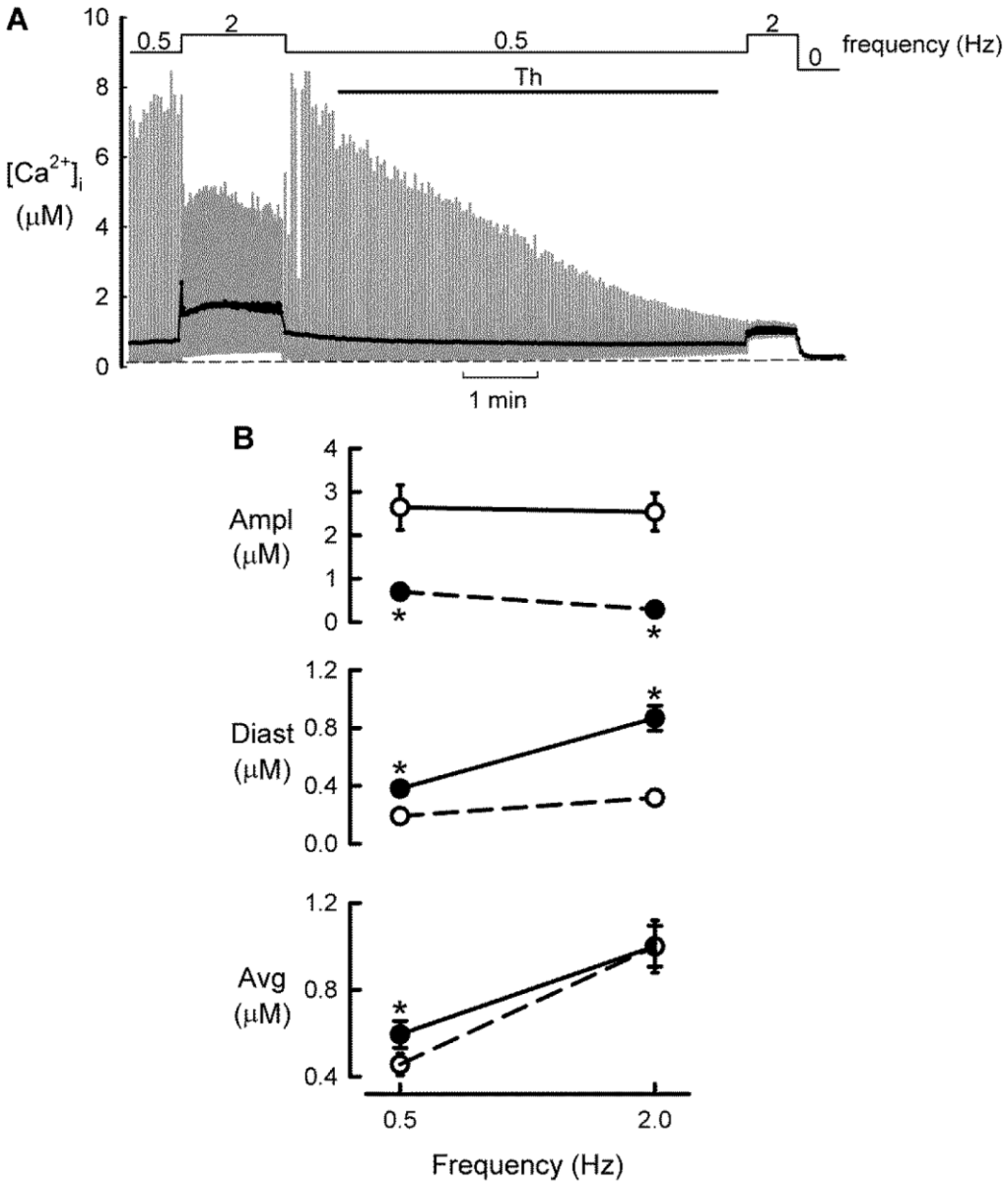


Figure 8. The effects of SERCA inhibition on $[Ca^{2+}]_i$. A, original record (rat ventricular myocyte). The cell was stimulated at the frequencies indicated and thapsigargin (1 μM) applied as shown. B,

mean data showing the effects of stimulation frequency on (from top to bottom): amplitude, diastolic and average $[Ca^{2+}]_i$. Reproduced from (Sankaranarayanan *et al.*, 2017)



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Competing Interests

None