



Calcium Buffering in the Heart in Health and Disease

ABSTRACT: Changes of intracellular Ca^{2+} concentration regulate many aspects of cardiac myocyte function. About 99% of the cytoplasmic calcium in cardiac myocytes is bound to buffers, and their properties will therefore have a major influence on Ca^{2+} signaling. This article considers the fundamental properties and identities of the buffers and how to measure them. It reviews the effects of buffering on the systolic Ca^{2+} transient and how this may change physiologically, and in heart failure and both atrial and ventricular arrhythmias, as well. It is concluded that the consequences of this strong buffering may be more significant than currently appreciated, and a fuller understanding is needed for proper understanding of cardiac calcium cycling and contractility.

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The importance of changes in intracellular calcium concentration in cardiac function needs little introduction (see^{1,2} for reviews). The systolic rise of ionized cytoplasmic calcium concentration ($[\text{Ca}^{2+}]_i$) activates contraction and regulates many sarcolemmal ion currents and, thereby, the electrophysiology of the cell; abnormal Ca^{2+} handling is implicated in the genesis of arrhythmias. Calcium is also a major factor in the control of gene expression. Work using fluorescent Ca^{2+} indicators has demonstrated how alterations of Ca^{2+} fluxes into the cytoplasm underlie the changes of contractility in health and disease. It is often overlooked, however, that only $\approx 1\%$ of cytoplasmic Ca^{2+} is free, with the remainder being bound to cytoplasmic buffers.³ Therefore, the properties of these buffers will potentially play as large a role as Ca^{2+} fluxes do in determining the size and kinetics of changes of $[\text{Ca}^{2+}]_i$.

Here we review recent progress in characterizing cytoplasmic buffers and their effects on the physiology of cardiac muscle, and disease mechanisms, as well. It is important to note that we also highlight the numerous areas where more work is required.

PROPERTIES OF INTRACELLULAR Ca^{2+} BUFFERS

Chemistry and Kinetics of Ca^{2+} Binding

Calcium (Ca^{2+}) has several chemical features that make it a ubiquitous second messenger.^{4,5} It forms a chemically active divalent cation in aqueous solution with an ionic radius larger than the other common divalent ion (Mg^{2+}) resulting in higher-affinity binding. The fact that its intracellular concentration is much lower than extracellular permits large changes of concentration because of sarcolemmal fluxes. Ca^{2+} binding alters the tertiary structure of proteins with consequences for their

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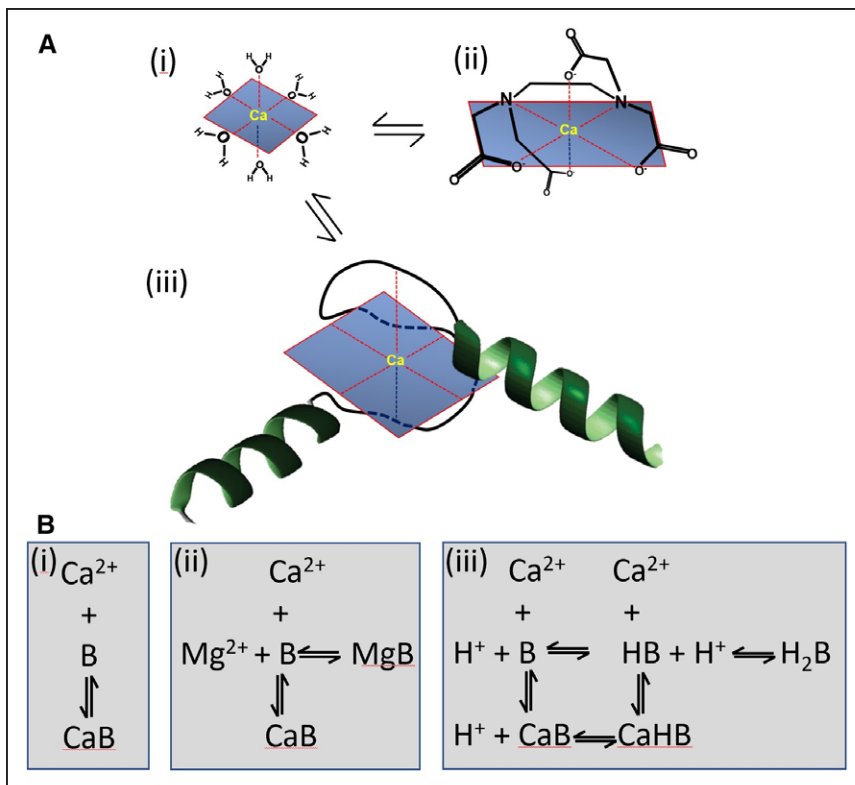


Figure 1. Chemistry of Ca²⁺ binding to buffers.

A, Schematics showing Ca²⁺ in various environments: water (i), bound to EDTA (ii), and bound to an EF hand (iii). **B**, Reaction schemes for Ca²⁺ binding to various buffers: simple binding (i), competition with Mg²⁺ (ii), and competition with protons (iii).

catalytic activity. This is a reciprocal interaction because binding also resists changes of the free concentration of an ion by acting as a buffer.

The formation of several coordinate bonds between single Ca²⁺ ions and ligands is known as chelation. The electrons for these bonds typically come from nitrogen or oxygen atoms, replacing water molecules in the solvation sphere of the Ca²⁺ ion with a series of bonds (usually 6) in a claw-like or chelation arrangement (Figure 1A). For example, EDTA is an organic chelator designed to bind divalent cations with very high affinity. The EF hand, a helix-loop-helix configuration, is the most common Ca²⁺ binding motif in proteins.⁶ EF hand sites generally occur in pairs, and their affinity for Ca²⁺ and Mg²⁺ depends on the amino acids used to form the coordinate bonds, and the surrounding protein environment, as well. For example, calmodulin has 4 EF hand domains: (1) a high-affinity site that is normally bound at resting Ca²⁺ concentrations with a dissociation constant (K_d) of ≈ 100 nmol/L, (2) 2 further binding sites on the C terminal with K_d values of ≈ 300 nmol/L.⁷ The fourth binding site has the lowest affinity ($K_d = 10$ $\mu\text{mol/L}$) and therefore binds negligible Ca²⁺ within the physiological range (0.1–1 $\mu\text{mol/L}$). It may have a role in controlling enzymes located near sarcoplasmic reticulum Ca²⁺ release sites in cardiac cells where $[\text{Ca}^{2+}]_i$ rises to ≈ 100 $\mu\text{mol/L}$.^{7,8}

The speed of the chelation reaction depends on its complexity (Table). The fastest binding occurs with small molecules (molecular weight <1000) such as BAPTA or

Ca²⁺ indicators (eg, Fura-2 or Fluo-3), with a forward rate constant that approaches the diffusion-controlled limit (minimally 10^8 mol⁻¹·L·s⁻¹).¹⁷ Ca²⁺ binding to the EF hand structure of the regulatory site of troponin C is slightly slower.⁹ With even more complex reaction schemes (Figure 1B) that involve displacement of ions (eg, H⁺ or Mg²⁺) before Ca²⁺ binding, the kinetics slows considerably. For example, Ca²⁺ binding to the chelator EGTA requires dissociation of protons from intermediate forms of the ligand, reducing the overall forward rate constant (Table).¹⁸ Different forms of the EF hand motif, such as the Mg²⁺ sites of myosin, troponin C (TnC), and parvalbumin, have high relative affinities for Mg²⁺ that result in significant Mg²⁺ bound under physiological conditions.¹⁹ The need for Mg²⁺ to dissociate as part of the equilibration results in a low apparent rate constant of Ca²⁺ binding.

Ca²⁺ Binding Sites in Cardiac Muscle

Based on previous work,^{3,11,20} the Table lists the major Ca²⁺ binding ligands, their estimated cytoplasmic concentrations, and dissociation constants (K_d) alongside estimates of the rate constants of Ca²⁺ binding. The ligands that bind appreciable amounts of Mg²⁺ under physiological conditions are grouped separately. The steady-state Ca²⁺ binding for several buffers as a function of $[\text{Ca}^{2+}]$ is shown in Figure 2Ai. The 2 major contributors to buffering are TnC and sarcoplasmic reticulum Ca²⁺ ATPase pump (SERCA).

Table. Concentration and Properties of the Major Cellular Buffers in Ventricular Myocytes

Buffers	Concentration $\mu\text{mol/L}$ Cell Cytoplasm	K_d $\mu\text{mol/L}$	On Rate $\text{mol}^{-1}\cdot\text{L}\cdot\text{s}^{-1}$
Ca buffers			
Troponin C (regulatory) ^{9,10}	70	0.6	3.27×10^7
SERCA ¹¹	47	0.6	$1.00\times 10^{8*}$
Calmodulin ³	24	7	3.4×10^7
Sarcolemma (low) ¹²	42	13	$1.0\times 10^{8*}$
Sarcolemma (high) ¹²	15	0.3	$1.0\times 10^{8*}$
Ca/Mg buffers			
Troponin C (Mg) ^{9,13}	140	0.0195	3.3×10^3
Myosin ⁹	140	4.62	9.6×10^4
ATP ¹⁴	5000	1200	1.9×10^6
Histidyl dipeptides ^{15,16}	20000	1000	6.1×10^6
Chemical probes			
BAPTA ¹⁷		0.178	5.0×10^8
EGTA ¹⁸ (pH 7.2)		0.180	2.3×10^6

The cellular buffers are organized in 2 groups: top, Ca buffers, and middle, Ca/Mg buffers. The properties of 2 chemical agents (BAPTA and EGTA) are shown below. For the cellular buffers, the first 2 columns show the buffer concentration and its K_d . The third column gives the value of the on rate constant for Ca²⁺ binding to the buffer. This has been calculated under standard conditions (initial free [Ca²⁺]_i 100 nmol/L, addition of 10 $\mu\text{mol/L}$ Ca²⁺ to 10 $\mu\text{mol/L}$ buffer). The value for calmodulin is an approximation to 1 binding site.¹¹ For the Mg/Ca buffers and EGTA, the K_d and rate constant have been corrected for the apparent value in the presence of a cellular Mg²⁺ of 0.5 mmol/L. SERCA indicates sarcoplasmic reticulum Ca²⁺ ATPase pump.

*Indicates rate constant was estimated based on diffusion limit.

Troponin C

TnC has 2 classes of Ca²⁺ binding sites (Figure 2A): (1) a single, lower-affinity, regulatory site that modulates myofibril activation and thence force; and (2) 2 high-affinity sites that can also bind Mg²⁺, the Mg²⁺ sites. There is ample evidence that the affinity for Ca²⁺ of the regulatory site changes in various situations. For example, acidification decreases the binding of Ca²⁺.²¹ Work using a fluorescent TnC showed that phosphorylation of troponin I, as occurs during β -adrenergic stimulation, shifts the relationship between fluorescence and [Ca²⁺]_i to higher [Ca²⁺]_i, indicating decreased Ca²⁺ affinity. A similar approach has shown that troponin and tropomyosin mutations affect Ca²⁺ affinity,²² and such mutations have been directly shown to affect Ca²⁺ buffering.^{23,24} It should, however, be noted that there are many circumstances (see below for discussion of heart failure) where the only available data are of a shift in the relationship between [Ca²⁺]_i and force. It is often not certain whether this shift results from a direct effect on Ca²⁺ binding or a subsequent step in the contraction mechanism.²⁵ For example, caffeine shifts the relationship to lower [Ca²⁺]_i but this effect is not accompanied by increased Ca²⁺ binding.²⁶ Finally, much less is known about the properties of the Mg²⁺ site on troponin than

the regulatory one. One issue, which also applies to other cellular buffers, is that studies of Ca²⁺ binding are generally performed in vitro using artificial solutions as opposed to cytoplasm. Given that many cellular constituents may affect the properties of this important buffer, it is important to characterize Ca²⁺ binding to the Mg²⁺ sites under more physiological conditions. These sites can be mutated, and normal contraction requires only 1 of the 2 Mg²⁺ sites.²⁷ It would be interesting to know the effects on cardiac function and Ca²⁺ cycling of the expected large decrease of Ca²⁺ buffering.

Sarcoplasmic Reticulum Ca²⁺ ATPase Pump

The inclusion of SERCA as a buffer emphasizes that it has 2 roles in decreasing cytoplasmic [Ca²⁺]_i.²⁸ Initial buffering by binding is followed by active sequestration. In rabbit ventricle, systole involves an increase of $\approx 60 \mu\text{mol/L}$ total Ca²⁺ resulting in a rise of free [Ca²⁺]_i of $\approx 0.6 \mu\text{mol/L}$. Because of the affinity of SERCA binding sites, $\approx 30 \mu\text{mol/L}$ binds immediately and, with a peak uptake rate of $\approx 200 \mu\text{mol}\cdot\text{L}^{-1}\cdot\text{s}^{-1}$, only ≈ 2 pump cycles are required to sequester the Ca²⁺ associated with a Ca²⁺ transient. This emphasizes the importance of the initial binding/buffering by SERCA in addition to its turnover in determining the rate of decay of the cytoplasmic Ca²⁺ transient.²⁹

Other Ligands

One important distinction is whether the buffers are immobile, or can diffuse. The Table gives values for the fixed sarcolemmal binding sites. The highly diffusible ATP binds Mg²⁺ and Ca²⁺ with moderately fast kinetics, but, although present at 5 mmol/L, its low affinity results in only a modest contribution to buffering. Other diffusible ligands include creatine phosphate and histidyl dipeptides that also bind Ca²⁺ and Mg²⁺. In heart, the predominant forms of this latter group of compounds include homocarnosine and anserine with a total concentration of $\approx 20 \text{ mmol/L}$.¹⁵ The affinities of Ca²⁺ and Mg²⁺ for these histidyl dipeptides are lower than that of ATP, and together they constitute the bulk of the diffusible Ca²⁺ buffers. One feature of diffusible Ca²⁺ buffers is their ability to increase the apparent diffusion coefficient of Ca²⁺ through diffusion of the Ca-bound form.³⁰ The histidyl dipeptides are also weak acids and contribute to the pH buffer power of the cytosol, thereby linking intracellular pH and Ca²⁺ buffering (see below).

Buffer Kinetics

The importance of the different kinetics of the major buffers is illustrated in Figure 2Bi. The amount of Ca²⁺ bound to the regulatory site of TnC lags slightly behind free [Ca²⁺]. The lag is much greater for the slower buffers (here the Mg²⁺ sites of myosin and TnC) and this is emphasized in the normalized data of Figure 2Bii. During a train of stimuli (Figure 2C), the slow kinet-

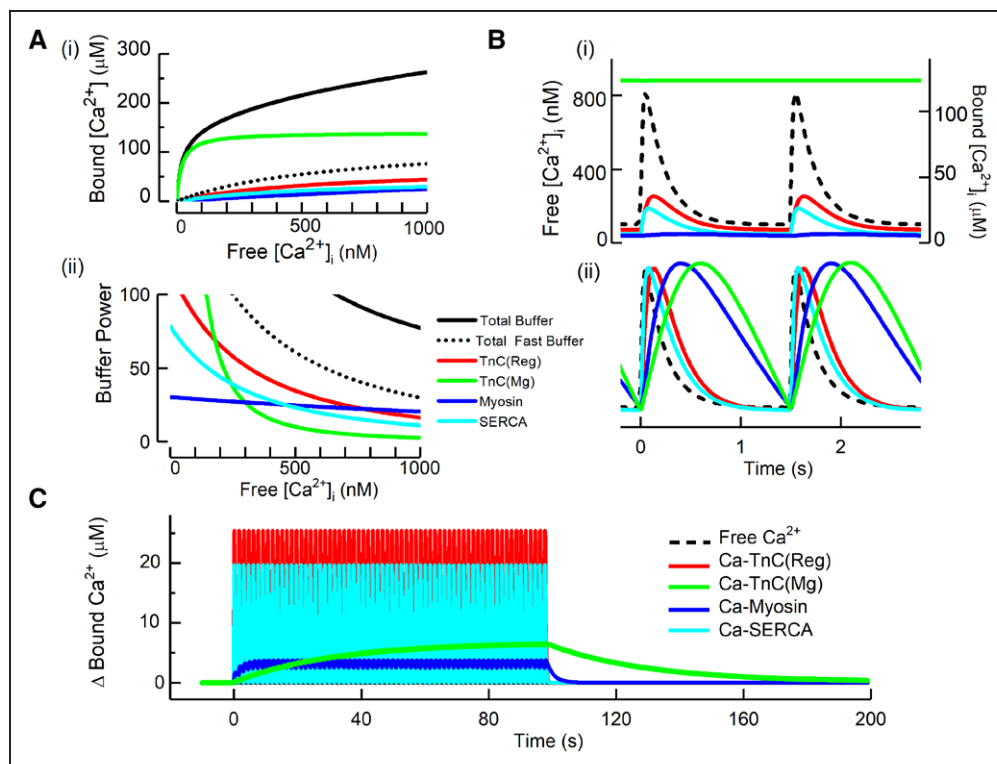


Figure 2. Properties of cellular cardiac Ca²⁺ buffers.

Ai, Steady-state Ca²⁺ buffering showing the dependence of bound on free Ca²⁺. The colored curves show the contributions of the 2 Ca²⁺ binding sites (regulatory [Reg] and Mg) of TnC, myosin, and SERCA. The solid black line shows the total, calculated as the sum of these components and the others listed in the Table. The dotted black line represents the sum of the fast buffers (top part of Table). **ii**, Buffer power (calculated as in equation 2) as a function of [Ca²⁺]_i. **B**, Time course of change of bound [Ca²⁺]_i in response to systolic Ca²⁺ transients applied at 1.5 Hz. **i**, Absolute levels of [Ca²⁺]_i. The dashed line shows free [Ca²⁺]_i and the colored traces show the concentration of the Ca²⁺-bound form of the various ligands. **ii**, Normalized concentrations to emphasize kinetics. **C**, The change of bound Ca²⁺ in response to a series of Ca²⁺ transients (not shown) applied at 1.5 Hz. SERCA indicates sarcoplasmic reticulum Ca²⁺ ATPase pump; and TnC, troponin C.

ics of these buffers results in a beat-to-beat increase of bound Ca²⁺. Even at 1.5 Hz, these 2 slow sites together accumulate a total of ≈10 μmol/L Ca²⁺ and, at higher rates, when diastolic [Ca²⁺]_i increases, greater binding is to be expected.

MEASUREMENT OF Ca²⁺ BUFFERING

As discussed above, buffering depends on the summed effects of a variety of Ca²⁺ binding molecules. It is often convenient to approximate this with a composite buffer value described by a single dissociation constant and ligand concentration. The simplest method is by titration. Solaro et al³¹ studied isolated cardiac myofilaments and calculated that about 22 μmol of Ca²⁺ per kg heart is required to produce 50% maximum contraction. This was accompanied by a rise of free Ca²⁺ of ≈1.4 μmol/L, indicating that the myofilaments alone can bind >90% of the total Ca²⁺. A subsequent approach, using cardiac homogenates, found that to raise free [Ca²⁺]_i to 1 μmol/L required 72 μmol/kg total Ca²⁺.³² Hove-Madsen and Bers³³ performed similar experiments using permeabilized cells. This removed complications of extracellular components and allowed study of mitochondrial and sarcoplasmic reticulum (SR)

buffering separately from cytoplasmic. They found that cytoplasmic buffering could be described by a K_d of 0.42 μmol/L, plus a much lower-affinity component ($K_d=79$ μmol/L).

The methods described above involve destruction of the cell membrane. It is also important to be able to measure buffering under physiological conditions. This was first done by depolarizing ventricular myocytes and measuring the total entry of calcium through the L-type Ca²⁺ current^{20,34} under conditions in which Ca²⁺ removal mechanisms were inhibited (see Figure 3A and 3B). Berlin et al³⁴ compared Ca²⁺ entry with the rise of [Ca²⁺]_i, giving a K_d of 0.96 μmol/L and a maximum buffer capacity of 123 μmol/L. A related method compared the entry of Ca²⁺ through sodium calcium exchange (NCX) with [Ca²⁺]_i, as estimated indirectly from changes of cell length.³⁷ A limitation of the method of Berlin et al is that it requires the irreversible SERCA inhibitor thapsigargin, precluding repeated measurements before and after other interventions. An alternative approach uses rapid application of caffeine to release calcium from the SR resulting in an abrupt increase of [Ca²⁺]_i which then decays as NCX removes Ca²⁺ from the cell. Integrating the NCX current gives a measure of the change of total Ca²⁺ concentration

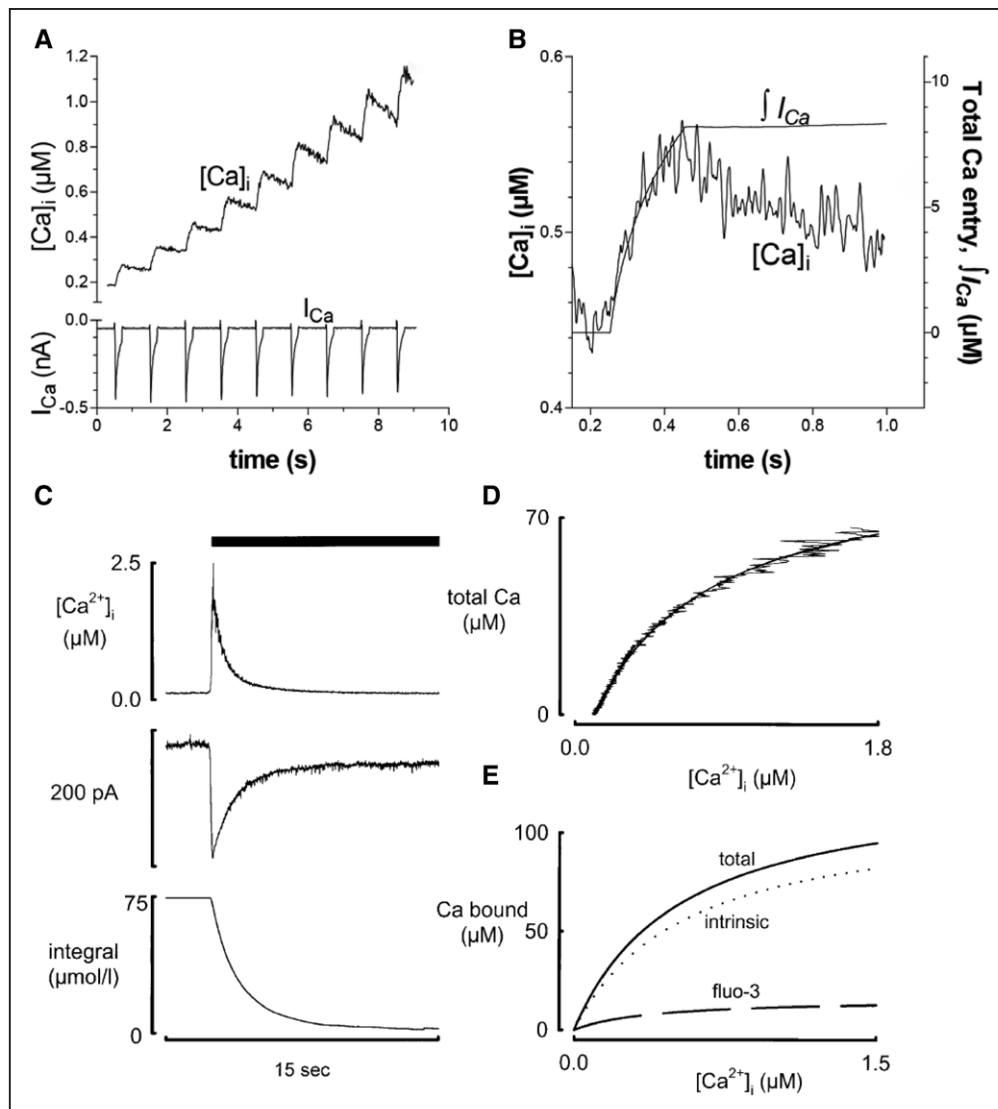


Figure 3. Measurement of buffering in intact cells.

A, Comparison of the effects of Ca²⁺ influx through the L-type Ca²⁺ channel (**bottom**) with the resulting increase of [Ca²⁺]_i. **B**, Relationship between the integral of the L-type Ca²⁺ current and the change of [Ca²⁺]_i. Ca²⁺ removal by SR, mitochondria, NCX, and PMCA were inhibited with thapsigargin, a mitochondrial uncoupler, Na-free solution and elevated external Ca²⁺ concentration, respectively. Figure reproduced from Bers³⁵ from an original article³⁴ with permission. Copyright © 2001, Kluwer Academic. **C**, Determination of Ca²⁺ buffering from the caffeine-evoked release of Ca²⁺ from the SR. Traces show (from top to bottom): [Ca²⁺]_i, NCX current, integral of current. Caffeine (10 mmol/L) was applied as shown by the bar. **D**, Relationship between total Ca²⁺ (estimated from the integral of NCX current) and [Ca²⁺]_i. **E**, Separation of buffering into total, the contribution from the Ca-sensitive indicator (fluo-3) and the calculated intrinsic buffering of the cytoplasm. Reproduced from Trafford et al.³⁶ NCX indicates sodium calcium exchange; PMCA, plasma membrane Ca²⁺ ATPase; and SR, sarcoplasmic reticulum.

that is compared continuously with the change of free [Ca²⁺]_i to characterize buffers³⁶ (Figure 3C through 3E). The caffeine response typically decays with a time constant of 1 to 2 s³⁸ so this cannot detect slower buffers. In ferret ventricular myocytes, this method gave a K_d of 0.59 μmol/L with a maximum capacity of 114 μmol/L cell equivalent to 175 μmol/L cytoplasm³⁶ and, in rat ventricular myocytes, a K_d of 0.49 μmol/L and a maximum capacity of 149 μmol/L cytoplasm.³⁹ This is stronger Ca²⁺ buffering than that found by Berlin et al.³⁴ This may be because, in part, the caffeine method includes buffering by SERCA because thapsigargin is not present. Consistent with this, addition of thapsigargin has been shown to decrease buffer power.⁴⁰

BUFFERING AND THE SYSTOLIC Ca²⁺ TRANSIENT

Alterations of buffering power affect the systolic Ca²⁺ transient and thence contraction. Incorporation of Ca-sensitive indicators has the side effect of increasing Ca²⁺ buffering, and this decreases systolic and increases diastolic force, and slows the rate of mechanical relaxation, as well.^{41,42} Subsequent work found a decrease of both the amplitude and rate constant of decay of the Ca²⁺ transient because, the higher the buffer power, the smaller the change of free [Ca²⁺]_i resulting from a given rate of Ca²⁺ pumping.⁴³ Adding exogenous buffer also decreases the rate of spontaneous beating of sinoatrial node cells,

presumably by decreasing the changes of [Ca²⁺]_i that contribute to pacemaker activity.⁴⁴ In recent years, much work has been done using transgenic animals that express calcium indicators. In principle, the additional buffering could be a concern, but it has been demonstrated that, at the concentrations expressed, this is not an issue.⁴⁵

The effect of increased buffering also depends on the kinetics of the added buffer. Although fast buffers simply slow the Ca²⁺ transient, slower buffers produce a biphasic decay. The initial, fast phase reflects the time taken for cytoplasmic Ca²⁺ to bind to the buffer with the slower phase depending on the kinetics of Ca²⁺ removal from the cytoplasm.⁴³

It should be noted that, in the steady state, averaged over the cardiac cycle, Ca²⁺ efflux must equal influx. This efflux is determined by [Ca²⁺]_i. If one assumes that Ca²⁺ efflux is proportional to [Ca²⁺]_i, then, in the steady state, the decrease of amplitude of the Ca²⁺ transient resulting from increased buffering must exactly balance the slowing of decay of the transient and increased diastolic level such that the average level of [Ca²⁺]_i is unaffected.^{46,47} Increasing stimulation rate will load cytoplasmic Ca²⁺ buffers (Figure 2C). An interruption of beating will result in this extra Ca²⁺ being taken up by the SR and then being available for release.²³ This may affect contractility and (see below) contribute to Ca-dependent arrhythmias. A more complicated question is what is the effect of increased buffering on SR Ca²⁺ content in the steady state? Experimental studies have found that adding exogenous cytoplasmic buffers decreases SR Ca²⁺.^{43,48} One explanation is that SERCA activity depends in a cooperative manner on [Ca²⁺]_i,⁴⁹ whereas NCX has a linear dependence.⁵⁰ The decreased amplitude of the systolic Ca²⁺ transient may therefore decrease SERCA activity more than NCX, leading to a net loss of SR Ca²⁺. Further studies are required to see if the decrease of SR content with increased buffering is a general phenomenon.

FACTORS THAT ALTER Ca²⁺ BUFFERING

Diastolic [Ca²⁺]_i

For a simple buffer, total ([Ca_T]) and free ([Ca²⁺]) are related by:

$$[Ca_T] = B_{max} \frac{[Ca^{2+}]}{K_d + [Ca^{2+}]} \quad (1)$$

where B_{max} is the total buffer concentration and K_d is the concentration of Ca²⁺ at which 50% of the buffer has Ca²⁺ bound. The upper graph of Figure 4A shows such relationships for 3 values of K_d .

Buffer power (β) is defined as the change of total Ca²⁺ divided by that of free Ca.

$$\beta = \frac{d[Ca_T]}{d[Ca^{2+}]} = B_{max} \frac{K_d}{([Ca^{2+}] + K_d)^2} \quad (2)$$

The individual contributions of the major individual buffers to the total buffer power are shown in Figure 2Aii. At [Ca²⁺]_i ≈ 100 nmol/L, the Mg²⁺ sites on TnC make the largest contribution, whereas, at >200 nmol/L, these are tending to saturation, and the regulatory site and SERCA contribute most. The lower graph of Figure 4A shows that buffer power has its highest value (equal to B_{max}/K_d) at low [Ca²⁺]_i and decreases as [Ca²⁺]_i increases. When [Ca²⁺]_i = K_d the buffer power is 30% of the value at 0.1 K_d and, at 2 K_d , it is only 13% of this level. Consequently, the greater the diastolic level of [Ca²⁺]_i, the larger will be the increase of [Ca²⁺]_i produced by a given release of total Ca from the SR.^{51,52} Some appreciation of the importance of this effect is provided by the buffer curve of Figure 4C. Because of the flattening of the buffer curve at elevated [Ca²⁺]_i, an increase of 60 μmol/L total [Ca²⁺] produces a larger increase of free [Ca²⁺] when applied from a higher diastolic [Ca²⁺]_i than from a lower. This is clear in the simulated transients of the upper graph of Figure 4D. An increase of diastolic [Ca²⁺]_i of only 100 nmol/L (from 50 to 150) increases systolic [Ca²⁺]_i by 500 nmol/L. Therefore, an increase of diastolic [Ca²⁺]_i alone can lead to an increase of systolic, which is predicted (see lower graph Figure 4D) to result in a large increase of developed force with little change of resting force. Finally, the decrease of buffer power at elevated [Ca²⁺]_i has also been suggested to account for a rapid initial rate of decay of the Ca²⁺ transient.⁵⁴

This consequence of changes of diastolic [Ca²⁺]_i will add to the inotropic effects of manoeuvres such as the addition of cardiac glycosides⁵⁵ or β-adrenergic stimulation⁵⁶ that can increase diastolic [Ca²⁺]_i. It is also a possible explanation for changes of the amplitude of the Ca²⁺ transient and force under the many conditions where there are no measurements of diastolic [Ca²⁺]_i. Testing this will require obtaining and comparing absolute measurements of [Ca²⁺]_i between cells or tissues from different animals or patients. There is a dearth of such measurements in the literature⁵⁷ because it is much easier to measure changes of fluorescence of a Ca²⁺-sensitive indicator than absolute levels of [Ca²⁺]_i. Properly calibrated measurements, ideally using ratio-metric indicators, are required.

Stimulation Rate

Repetitive stimulation will load slower Ca buffers (Figure 2C). This will decrease buffer power and might therefore increase the rise of [Ca²⁺]_i produced by a given increase of total cytoplasmic Ca²⁺, contributing to the inotropic effects of increased rate.⁵² This effect is analogous to that discussed above for elevated diastolic [Ca²⁺]_i, but the slow kinetics result in a memory so that, following a change of rate, the effects on systolic [Ca²⁺]_i and thence on the action potential duration may

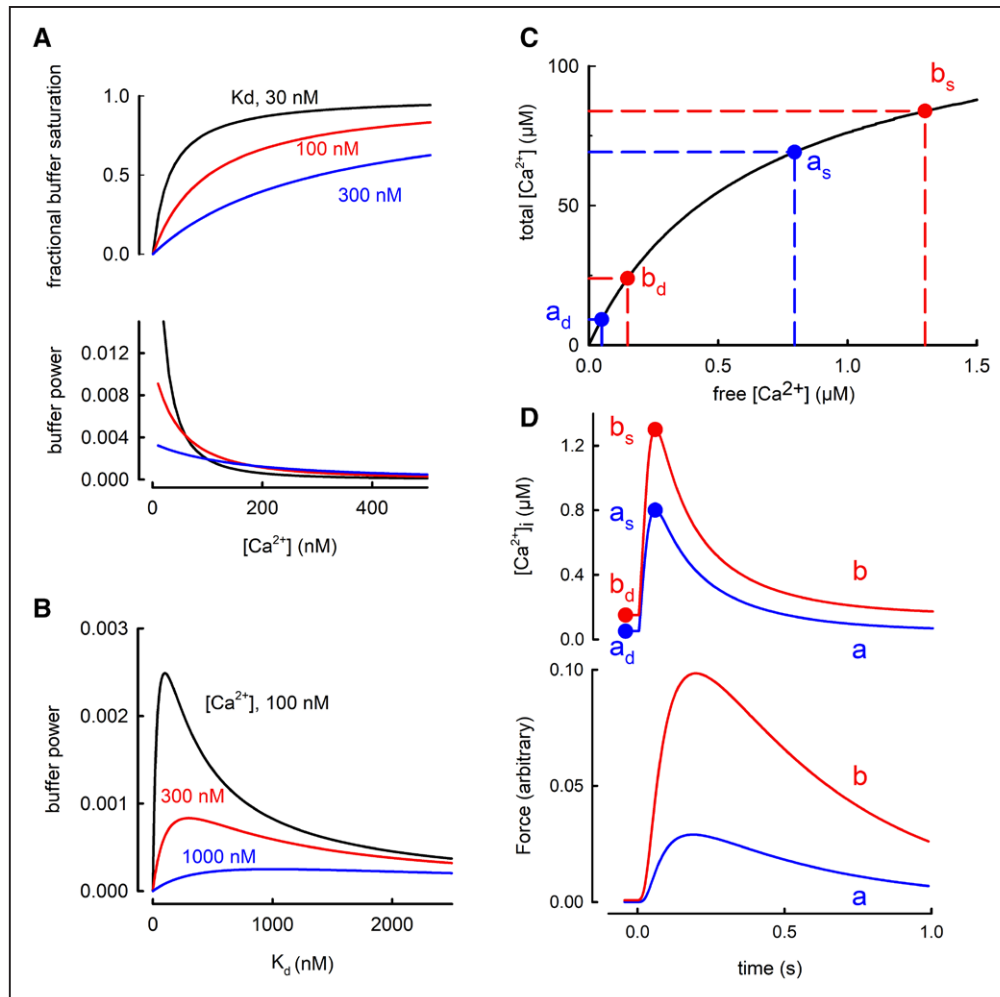


Figure 4. Effects of [Ca²⁺]_i and buffer K_d on buffer power.

A, Effects of [Ca²⁺]_i. The top graph shows fractional saturation of a single buffer as a function of [Ca²⁺]_i. Curves are shown for 3 different values of K_d . The lower graph shows calculated buffer power (change of total Ca²⁺/change of [Ca²⁺]_i) as a function of [Ca²⁺]_i for these values of K_d . Colors correspond to those above. **B**, Dependence of buffer power on K_d at the 3 values of [Ca²⁺]_i indicated. **C**, The effects of a small increase of diastolic [Ca²⁺]_i on the change of systolic [Ca²⁺]_i produced by the addition of a fixed amount of total [Ca²⁺]. The buffer curve (fast buffers only) shows 2 levels of diastolic [Ca²⁺]_i indicated as a_d (50 nmol/L) and b_d (150 nmol/L). The systolic levels (respectively, a_s and b_s) are obtained by adding 60 μmol/L total [Ca²⁺] resulting in a larger increase of systolic [Ca²⁺]_i in **b** compared with **a**. **D**, Simulation of the effect of adding and removing 60 μmol/L total Ca²⁺ with kinetics designed to represent a Ca²⁺ transient. Labels correspond to points in **C**. **Upper**, simulated Ca²⁺ transients; **Lower**, predicted force responses calculated using a published model.⁵³

outlast those of diastolic [Ca²⁺]_i. Such effects may also contribute to the slow effects of changes of rate on parameters such as action potential duration.⁵⁸

Buffer K_d

Equation 2 (Figure 4A) shows that, at lower values of [Ca²⁺]_i, buffer power is greater the lower the value of K_d because this results in stronger Ca²⁺ binding. In contrast, at higher [Ca²⁺]_i, the lower the K_d , the less the buffer power as the buffers become saturated (see²³ for experimental demonstration). Figure 4B shows the biphasic dependence of buffer power on K_d with the maximum being reached when $K_d=[Ca^{2+}]_i$. Therefore, increasing buffer affinity will increase buffering at diastolic levels of [Ca²⁺]_i but decrease it at peak systolic ones.

One issue that has received no attention is whether Ca²⁺ buffering is the same in all cells in the ventricle. Given the regional differences of expression of other proteins including pumps⁵⁹ and channels,⁶⁰ heterogeneity of buffering would not be surprising. Likewise, possible variations of Ca²⁺ buffering between individuals, because of mutations and polymorphisms, do not appear to have been considered.

PHYSIOLOGICAL MODULATION OF BUFFERING

β-Adrenergic Stimulation

The 2 major Ca²⁺ buffers, TnC and SERCA, are regulated by the phosphorylation of troponin I and phospholamban, respectively, resulting in increased affinity

of Ca²⁺ for SERCA⁶¹ and decreased affinity of Ca²⁺ for TnC.⁶² One might therefore expect that β-adrenergic stimulation would alter the buffer power. Experimental measurements, however, found no such effect,⁴⁰ possibly because of 2 opposing factors: phosphorylation increases the affinity of Ca²⁺ binding to SERCA, but lowers it for troponin. If the K_d values are above the range of $[Ca^{2+}]_i$ considered, these effects will respectively increase and decrease buffer power (Figure 4A). Subsequent experiments, performed on transgenic mice in which either troponin could not be phosphorylated or lacking phospholamban, found the expected increase and decrease, respectively, of buffer power on phosphorylation. Further work is required to investigate the possibility that, in other species, the balance is less exact, and, therefore, phosphorylation may have a net effect on buffer power. As mentioned above, it should also be noted that the effects of a change of Ca²⁺ affinity on buffer power will depend on the range of $[Ca^{2+}]_i$ under investigation.

Effects of Changes of pH on Buffering

Many Ca²⁺ buffers can bind protons as an alternative to Ca²⁺ ions. Direct measurements have shown that acidification decreases Ca²⁺ binding to troponin.²¹ Therefore, acidification will decrease the affinity for Ca²⁺ with a decrease of Ca²⁺ buffering power predicted at values of $[Ca^{2+}]_i$ below the K_d (Figure 4B). It is surprising that intracellular acidification had no effect on Ca²⁺ buffering.⁶³ We suggest that this may occur because, although a decrease of Ca²⁺ affinity of low-affinity buffers will decrease buffer power, decreased affinity of very-high-affinity buffers will increase their contribution to buffering. Acidification has been shown to increase resting $[Ca^{2+}]_i$ in rat ventricular myocytes, an effect attributed to displacement of Ca²⁺ from buffers.¹⁵ It is not clear, however, why such displacement should produce the observed maintained increase of $[Ca^{2+}]_i$; one would expect a transient increase that decays back to baseline as Ca²⁺ is pumped out of the cell. It may result from the inhibition of Ca²⁺ efflux on NCX by acidification.⁶⁴ If this is the case then the maintained effect on $[Ca^{2+}]_i$ is presumably a consequence of the NCX effect and not of altered buffering. This question could be resolved by directly measuring the effects of pH on NCX activity.

CARDIAC DYSFUNCTION AND Ca²⁺ BUFFERING

Atrial Buffering, Fibrillation, and Failure

The total concentration of Ca²⁺ buffers in rat atrial myocytes has been reported to be about 3 times greater than in ventricular myocytes with no difference in apparent K_d ,⁶⁵ possibly because of higher SERCA expres-

sion in the atrium than in the ventricle. Changes in Ca²⁺ buffering have been suggested to be important in both normal and abnormal atrial function. For example, in sheep atria, buffer power increases with age because of an increase of Ca²⁺ affinity of the buffers.⁶⁶ This decreased both the amplitude and rate of decay of the systolic Ca²⁺ transient. Atrial myocytes from many species, including rabbits and cats, have few or no t-tubules (⁶⁷ for review) and the systolic Ca²⁺ transient begins at the periphery of the cell and then propagates toward the center.^{68,69} Increasing Ca²⁺ buffering by incorporation of EGTA can prevent this propagation.⁷⁰ A modeling study also predicted this inhibitory effect of high buffer concentrations but pointed out that lower concentrations of mobile buffers such as ATP facilitate propagation.³⁰ Greiser et al⁷¹ investigated the effects of rapid atrial pacing in rabbits to mimic the effects of atrial fibrillation. This resulted in a 2- to 3-fold increase of buffering power, at least in part, because of decreased phosphorylation of troponin I which was accompanied by (Figure 5A) decreased centripetal propagation. Evidence for a causal link between increased buffering and decreased propagation was provided by showing that incorporation of BAPTA to increase buffering mimicked the effect on propagation. The effects of rapid pacing to induce heart failure have also been studied on sheep atrial myocytes where a decrease of buffer power was observed.⁷⁴ This was accompanied by a decrease of the amplitude of the central calcium transient attributed to the loss of transverse tubules rather than a change of buffering.⁷⁵ A similar decrease of calcium buffering in the sheep has been observed during atrial fibrillation where it was suggested to lead to arrhythmogenic Ca²⁺ waves, thereby contributing to atrial fibrillation.⁷⁶ More work is required on changes of atrial Ca²⁺ buffering and their importance in atrial function. Finally, it is worth noting that the atrial studies reviewed above could not exclude the effects of small changes of end-diastolic $[Ca^{2+}]_i$ on the measured buffer power.

Ca²⁺ Buffering and Heart Failure

Ca²⁺ buffering is unaffected by pacing-induced heart failure in both dogs⁷⁷ and sheep.⁷⁸ In contrast, in samples from human ventricle, the Ca²⁺ sensitivity of contraction was increased in dilated cardiomyopathy, possibly because of the decreased phosphorylation of troponin I.⁷⁹ Increased Ca²⁺ sensitivity was also found in canine dilated cardiomyopathy⁸⁰ and mouse infarct models.^{81,82} As mentioned earlier, changes of Ca²⁺ sensitivity of contraction do not necessarily indicate altered Ca²⁺ binding and buffering; direct measurements of Ca²⁺ binding are therefore required. Increased myofilament Ca sensitivity by itself will decrease cardiac relaxation and thereby contribute to diastolic heart failure. In addition, any consequential increase of Ca²⁺ buffering

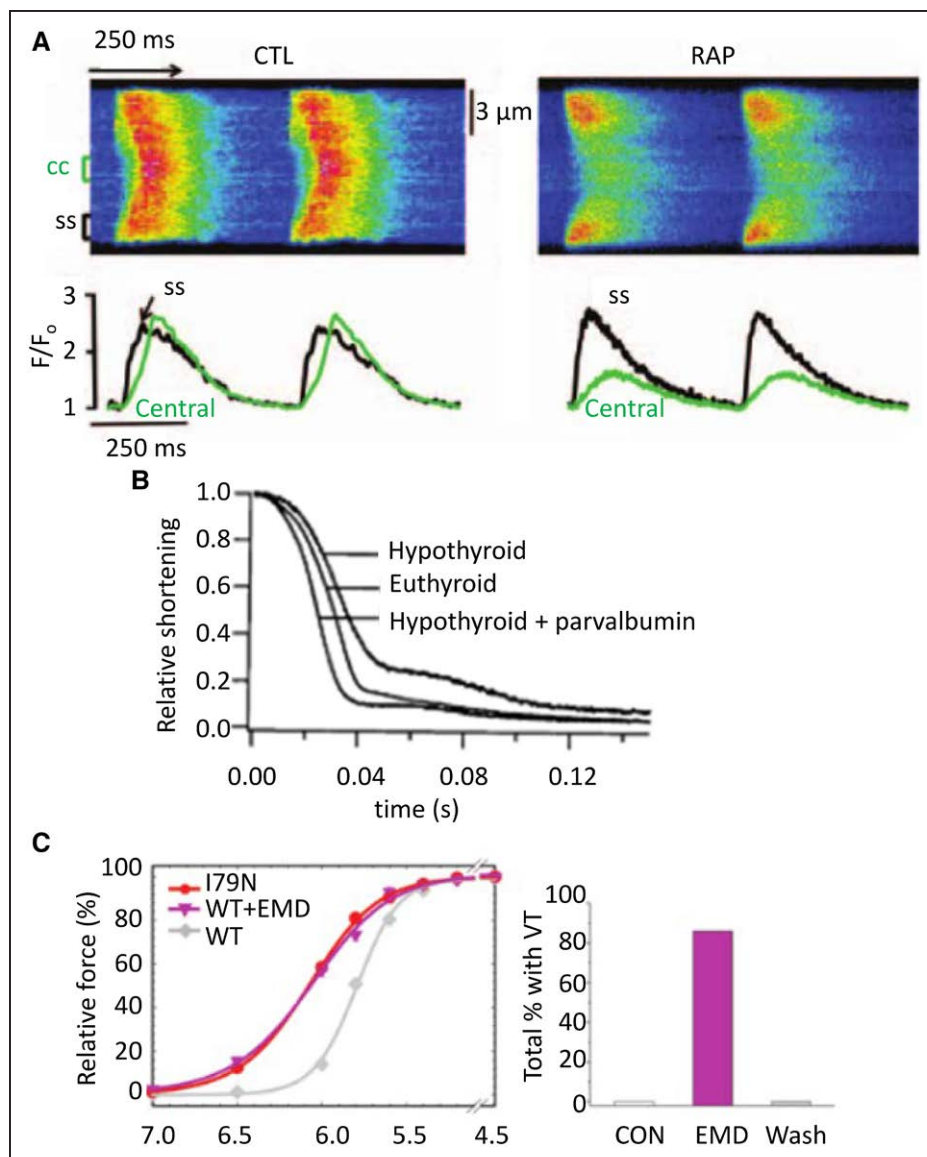


Figure 5. Effects of altered buffer power.

A, Abolition of centripetal propagation of the Ca²⁺ transient by rapid atrial pacing (RAP). Upper traces are linescan images. Lower records show [Ca²⁺]_i measured at surface of cell (black) and in center (green). **Left**, Records from control. **Right**, Records after rapid stimulation. Reproduced from.⁷¹ **B**, Relaxation of contraction in isolated rat myocytes. Records show control (euthyroid), hypothyroid, and hypothyroid with parvalbumin expressed by gene transfer. Reproduced from.⁷² **C**, **Left**, comparison of the effects of the I79N mutation of troponin T with that of the Ca²⁺ sensitizing agent EMD 57033 on the relationship between pCa and force. **Right**, Effects of EMD 57033 on the occurrence of ventricular tachycardia (VT). Reproduced from Greiser et al.⁷³

will slow the decay of [Ca²⁺]_i, worsening relaxation. In contrast to the data discussed above, either the induction in rats of pressure overload–induced left ventricular hypertrophy or heart failure following myocardial ischemia resulted in a decreased Ca²⁺ sensitivity for activation of contraction, an effect attributed to alterations in troponin.⁸³ Some of the controversies in this area have been reviewed.⁸⁴ As far as myocardial ischemia is concerned, it is well known that troponin is lost from the heart and, indeed, the appearance of troponin I and troponin T in plasma is diagnostic of cardiac damage. Troponin release has also been detected in myocardium in conditions not associated with obvious cellular degeneration, but this only represents a small fraction

(≈3%) of the total troponin⁸⁵ and will not therefore significantly affect cellular buffering.

Finally, it should be noted that many studies of heart failure find a decrease of SERCA expression.⁸⁶ We speculate that the consequent decrease of Ca²⁺ buffering would compensate in those situations where an increase of myofilament buffering is expected and worsen where there is a decrease. Again, it will be important to repeat these studies of heart failure while measuring buffering directly.

It is also important to reemphasize the potential effects (Figure 4C) of changes of diastolic [Ca²⁺]_i and, thence, of buffering power on the amplitude of the calcium transient. A major problem here is the paucity

of measurements of diastolic [Ca²⁺]_i. It is essential that studies on heart failure ask the simple question: how accurately has diastolic [Ca²⁺]_i been measured and can it be excluded that changes (for example, between animals or in disease) account for the observed changes of systolic [Ca²⁺]_i?

Work from Metzger and colleagues has demonstrated that changes of Ca²⁺ buffering may not simply be involved in the development and consequences of heart failure, but may also be used to treat it. They suggested that impaired relaxation in heart failure could be ameliorated by adding intracellular buffers. They noted that fast Ca²⁺ buffers slow both the rise and fall of [Ca²⁺]_i and decrease the amplitude of the Ca²⁺ transient, and, instead, they advocated the use of parvalbumin, a skeletal muscle Ca²⁺ buffer. This has the important property that it binds Ca²⁺ slowly because Mg²⁺ has to dissociate first and, therefore, there is little attenuation of the peak Ca²⁺ transient. It will bind Ca²⁺ during diastole thereby improving diastolic performance. Incorporation of α -parvalbumin was shown to accelerate the decay of [Ca²⁺]_i with no effect on peak [Ca²⁺]_i and (see Figure 5B) also reversed the slowing of relaxation produced by experimental hypothyroidism⁷² and in the Dahl salt-sensitive rat model of diastolic dysfunction.⁸⁷ Subsequent work has turned to altering the structure and thence the relative Ca²⁺ and Mg²⁺ affinities of parvalbumin analogs to improve the effects.^{88,89} In general, these effects of parvalbumin highlight the potential importance of endogenous slow buffers such as the Mg²⁺ site of TnC and myosin.

Ca²⁺ Buffering and Hypertrophic Cardiomyopathy

Several studies have examined the molecular basis of familial hypertrophic cardiomyopathy (FHC). Much of this work involves the effects of mutations in thin filament proteins such as troponin and tropomyosin, which are among the causes of FHC. Robinson et al²² showed that mutations causing hypertrophic cardiomyopathy increased the binding affinity of Ca²⁺ to myofilaments (as assessed with a fluorescent troponin) and presumably therefore Ca²⁺ buffering. They proposed that alterations of buffering might lead to pathological changes of the Ca²⁺ transient. Troponin mutations were subsequently investigated in a mouse model of the related condition of restrictive cardiomyopathy and the predicted decreased amplitude and slowed decay of the Ca²⁺ transient observed.^{23,90} In addition, myofilament Ca²⁺ sensitization with EMD 57033 mimicked the effects of troponin T mutations on Ca²⁺ buffering and the Ca²⁺ transient.²³ A recent study used adenovirus to infect isolated myocytes with troponin or tropomyosin mutations and, again, found

an increase of diastolic [Ca²⁺]_i.²⁴ Although the above results would be expected from an increase of buffering power, it has been reported that there is a decrease of SERCA expression that may also contribute.⁹¹ This study also found that the late Na⁺ current inhibitor ranolazine abolished the slowing of decay of the Ca²⁺ current. Although no data are available, it seems unlikely that ranolazine would affect Ca²⁺ buffering. It may therefore be that some of the effects of thin filament mutations are directly attributable to Ca²⁺ buffering, and others are a secondary consequence of the resulting heart failure, possibly attributable to decreased SERCA.

As mentioned in an earlier section, the Mg²⁺ sites on troponin are important contributors to buffering at low [Ca²⁺]_i. It is therefore interesting that one of the mutations associated with FHC (D145E) greatly decreases the affinity of Ca²⁺ binding to these sites.⁹² At first sight, this might appear to contrast with the association between FHC and the increased affinity reviewed above. These observations may be reconciled by noting that a decrease in affinity of the very-high-affinity Mg²⁺ TnC sites will actually increase Ca²⁺ buffering power in the systolic range of [Ca²⁺]_i.

Ca²⁺ Buffering and Arrhythmias

Ventricular arrhythmias constitute a major cause of death in FHC.⁹³ The Knollmann group has investigated the underlying mechanisms in transgenic mice. Incorporation of mutations in troponin T or tropomyosin led to ventricular tachycardia. These mutations also sensitized the contractile machinery to activation by Ca²⁺ (Figure 5C) with those that produced the greatest incidence of ventricular tachycardias and arrhythmias having the greatest Ca-sensitizing effect.⁷³ A causal link between Ca sensitization and arrhythmogenesis was provided by showing both that EMD 57033 caused arrhythmias and the contractile uncoupler blebbistatin decreased both Ca sensitivity of the contractile machinery and arrhythmia susceptibility. These arrhythmias were accompanied by a shortening and triangulation of the action potential, and electric repolarization alternans, as well (see below). Subsequent work using myocytes derived from human-induced pluripotent stem cells reproduced these effects of increased Ca²⁺ buffering by myofilaments on action potential shape and suggested that the shortened, triangulated action potential could be attributable to increased buffering decreasing the amplitude of the systolic Ca²⁺ transient and thereby the inward (depolarizing) NCX current.⁹⁴ Although this is an attractive explanation, it is also worth noting that (see above), as well as decreasing the amplitude of the Ca²⁺ transient, increased buffering slows decay, making it harder to predict the net effect of increased buffering on NCX

current. Another article showed that, when regular pacing was terminated by a pause, the next Ca²⁺ transient was larger than control and this effect was more prominent in troponin T mutations that sensitize to activation by Ca²⁺.²³ This effect was attributed to a higher cell Ca²⁺ content in the mutant during stimulation, with the excess Ca²⁺ being taken up by the SR such that release after a pause results in a prolonged action potential, increasing the probability of an arrhythmogenic early afterdepolarization. Any increase of diastolic [Ca²⁺]_i and consequent decrease of buffer power may also increase the rise of [Ca²⁺]_i caused by release from the SR. In the work reviewed above, the increase of buffering was a consequence of genetic changes. Similar increases of myofilament Ca²⁺ sensitivity have been reported following myocardial infarction⁸¹ where manoeuvres that decrease Ca²⁺ sensitivity were found to abolish ventricular tachycardia following pauses of stimulation. As reviewed above, an increase of Ca²⁺ buffering is proarrhythmogenic. It has also been shown, however, that addition of the buffer EGTA can prevent the propagation of arrhythmogenic Ca²⁺ waves,⁹⁵ and therefore the net effect may be more complicated.

The induction of alternans of the action potential duration by increased Ca²⁺ buffering attributable to thin filament mutations⁷³ may be a consequence of the slowed decay of the Ca²⁺ transient, resulting in incomplete recovery at the time of the next stimulus at increased rates. This would be analogous to the idea that the increased propensity of endocardium in comparison with epicardium to alternans is associated with a more slowly decaying Ca²⁺ transient because of lower SERCA expression.⁵⁹ In contrast, a modeling study has predicted that increasing cytoplasmic Ca²⁺ buffering should decrease the probability of alternans occurring by decreasing the probability that Ca²⁺ released from the SR induces further Ca²⁺ release from neighboring release sites.⁹⁶ This is consistent with the experimental demonstration, in whole mouse hearts, that addition of the buffer EGTA decreased the occurrence of alternans⁴⁸ and may be related to the experimental observation that increasing cytoplasmic Ca²⁺ buffering decreases the frequency of propagating Ca²⁺ waves⁹⁷ and makes Ca²⁺ sparks terminate earlier.⁹⁸ Further work is clearly required in understanding the relationship between Ca²⁺ buffering and alternans.

Why Do Cells Have Ca²⁺ Buffers?

A high level of Ca²⁺ buffering is not unique to cardiac myocytes. For example, ≈99% of the Ca²⁺ entering chromaffin cells binds to cytoplasmic buffers.⁹⁹ The presence of Ca²⁺ buffers means that much larger movements of total Ca²⁺ are required to produce a given change of [Ca²⁺]_i. Given that calcium move-

ments account for up to 30% of the total energy consumption of the heart,¹⁰⁰ one might wonder why evolution has resulted in such strong buffering. There are several explanations. (1) It may be an inescapable consequence of the fact that using Ca²⁺ as a second messenger requires high concentrations of Ca²⁺ binding proteins, for example, to activate contraction. (2) A high Ca²⁺ buffering may stabilize Ca²⁺ signaling by stopping an abnormal increase of [Ca²⁺]_i in one part of a cell propagating throughout the cell. In this context, it is worth noting that (Figure 2Aii) the dependence of buffer power on [Ca²⁺]_i means that the buffer power is much lower in systole than diastole. This may help Ca²⁺ release during systole, but protect against it in diastole. (3) The need for buffering may relate to the low intracellular concentration of calcium. A diastolic concentration of 100 nmol/L equates to 6×10¹⁶ ions per liter corresponding to a mean distance between ions of 0.25 μm. Soeller and Cannell⁸ have modeled Ca²⁺ fluxes into the space between the transverse tubule and SR (dyad). They calculated that, at a concentration of 100 nmol/L, each dyad would contain between 0.007 and 0.028 Ca²⁺ ions. At 10 μmol/L, there will be between 0.7 and 2.8 ions. This would make it impossible to control [Ca²⁺]_i in a stable manner because a single Ca²⁺ transported into or out of the space would result in an enormous fractional change of [Ca²⁺]_i. As pointed out previously by Bers,³⁵ at such low concentrations, chance will determine whether a transporter interacts with an ion. In contrast, if total Ca²⁺ is 100 times the free then there will be between 70 and 280 ions per cleft. (4) If troponin was the only buffer, then virtually all the total Ca²⁺ would be bound to troponin irrespective of its K_d. This would make it impossible to change force by altering K_d because this requires other buffers to take up a fraction of the total Ca.

CONCLUSIONS

The concentration of buffered calcium in cytoplasm is 2 orders of magnitude greater than that of the free concentration, and, therefore, the buffers have an enormous effect on calcium signaling. There is a need for more work investigating whether changes of buffer properties, either directly or secondary to changes of diastolic [Ca²⁺]_i, contribute to alterations of calcium handling and contractility. The limited human data reviewed above and extrapolated from animal models argue that changes of Ca²⁺ buffering are important in determining both inotropy and proarrhythmic status in conditions such as cardiomyopathies (dilated cardiomyopathy and hypertrophic cardiomyopathy) and ischemic heart failure in both health and disease. Clarification will require more work on human tissue.

ARTICLE INFORMATION

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