Circulation

IN DEPTH

Calcium Buffering in the Heart in Health and Disease

Godfrey L. Smith, PhD
David A. Eisner, DPhil

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.

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 ($[Ca^{2+}]_i$) 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 ≈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 $[Ca^{2+}]_i$.

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

Key Words: arrhythmias, cardiac ■ buffers ■ calcium ■ heart failure

https://www.ahajournals.org/journal/circ

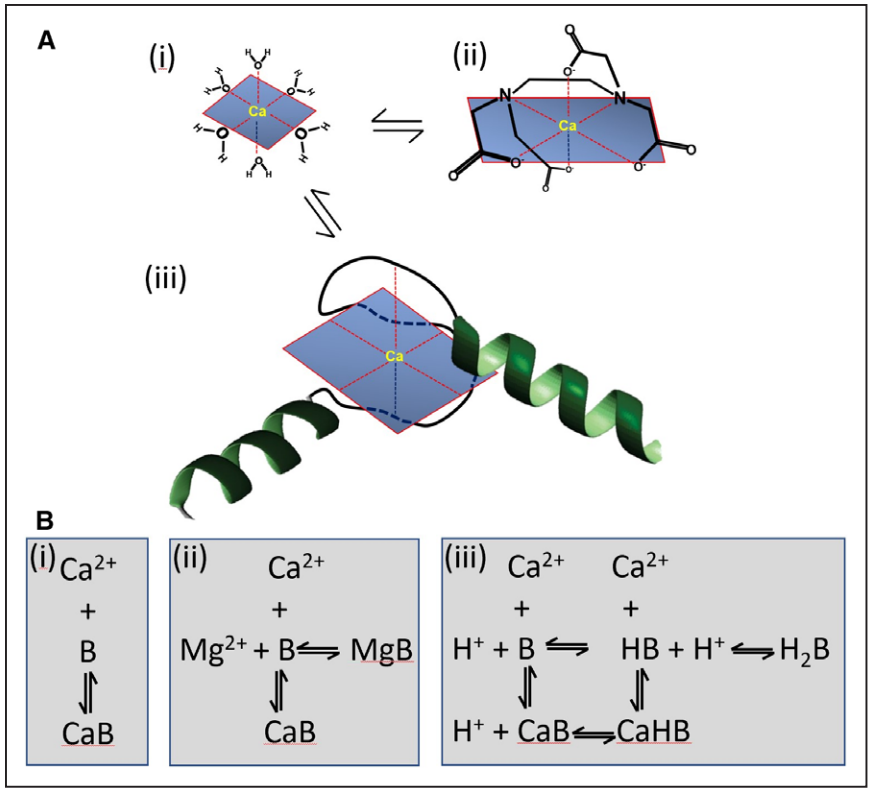

Figure 1. Chemistry of Ca^{2+} binding to buffers.
A, Schematics showing Ca^{2+} in various environments: water (**i**), bound to EDTA (**ii**), and bound to an EF hand (**iii**). **B**, Reaction schemes for Ca^{2+} binding to various buffers: simple binding (**i**), competition with Mg^{2+} (**ii**), 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 Ca^{2+} 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 Ca^{2+} 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 Ca^{2+} binding motif in proteins.[6] EF hand sites generally occur in pairs, and their affinity for Ca^{2+} and Mg^{2+} 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 Ca^{2+} 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 μmol/L) and therefore binds negligible Ca^{2+} within the physiological range (0.1–1 μmol/L). It may have a role in controlling enzymes located near sarcoplasmic reticulum Ca^{2+} release sites in cardiac cells where $[Ca^{2+}]_i$ rises to ≈100 μmol/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 Ca^{2+} indicators (eg, Fura-2 or Fluo-3), with a forward rate constant that approaches the diffusion-controlled limit (minimally 10^8 $mol^{-1}\cdot L\cdot s^{-1}$).[17] Ca^{2+} 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 Mg^{2+}) before Ca^{2+} binding, the kinetics slows considerably. For example, Ca^{2+} 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 Mg^{2+} sites of myosin, troponin C (TnC), and parvalbumin, have high relative affinities for Mg^{2+} that result in significant Mg^{2+} bound under physiological conditions.[19] The need for Mg^{2+} to dissociate as part of the equilibration results in a low apparent rate constant of Ca^{2+} binding.

Ca^{2+} Binding Sites in Cardiac Muscle

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

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

Buffers	Concentration μmol/L Cell Cytoplasm	K_d μmol/L	On Rate $mol^{-1}·L·s^{-1}$
Ca buffers			
Troponin C (regulatory)[9,10]	70	0.6	$3.27×10^7$
SERCA[11]	47	0.6	$1.00×10^8$*
Calmodulin[3]	24	7	$3.4×10^7$
Sarcolemma (low)[12]	42	13	$1.0×10^8$*
Sarcolemma (high)[12]	15	0.3	$1.0×10^8$*
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] (pH7.2)		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 Ca^{2+} binding to the buffer. This has been calculated under standard conditions (initial free $[Ca^{2+}]$ 100 nmol/L, addition of 10 μmol/L Ca^{2+} to 10 μmol/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 Mg^{2+} of 0.5 mmol/L. SERCA indicates sarcoplasmic reticulum Ca^{2+} ATPase pump.

*Indicates rate constant was estimated based on diffusion limit.

Troponin C

TnC has 2 classes of Ca^{2+} 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 Mg^{2+}, the Mg^{2+} sites. There is ample evidence that the affinity for Ca^{2+} of the regulatory site changes in various situations. For example, acidification decreases the binding of Ca^{2+}.[21] Work using a fluorescent TnC showed that phosphorylation of troponin I, as occurs during β-adrenergic stimulation, shifts the relationship between fluorescence and $[Ca^{2+}]_i$ to higher $[Ca^{2+}]_i$, indicating decreased Ca^{2+} affinity. A similar approach has shown that troponin and tropomyosin mutations affect Ca^{2+} affinity,[22] and such mutations have been directly shown to affect Ca^{2+} 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 $[Ca^{2+}]_i$ and force. It is often not certain whether this shift results from a direct effect on Ca^{2+} binding or a subsequent step in the contraction mechanism.[25] For example, caffeine shifts the relationship to lower $[Ca^{2+}]_i$ but this effect is not accompanied by increased Ca^{2+} binding.[26] Finally, much less is known about the properties of the Mg^{2+} site on troponin than the regulatory one. One issue, which also applies to other cellular buffers, is that studies of Ca^{2+} 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 Ca^{2+} binding to the Mg^{2+} sites under more physiological conditions. These sites can be mutated, and normal contraction requires only 1 of the 2 Mg^{2+} sites.[27] It would be interesting to know the effects on cardiac function and Ca^{2+} cycling of the expected large decrease of Ca^{2+} buffering.

Sarcoplasmic Reticulum Ca^{2+} ATPase Pump

The inclusion of SERCA as a buffer emphasizes that it has 2 roles in decreasing cytoplasmic $[Ca^{2+}]_i$.[28] Initial buffering by binding is followed by active sequestration. In rabbit ventricle, systole involves an increase of ≈60 μmol/L total Ca^{2+} resulting in a rise of free $[Ca^{2+}]_i$ of ≈0.6 μmol/L. Because of the affinity of SERCA binding sites, ≈30 μmol/L binds immediately and, with a peak uptake rate of ≈200 $μmol·L^{-1}·s^{-1}$, only ≈2 pump cycles are required to sequester the Ca^{2+} associated with a Ca^{2+} 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 Ca^{2+} 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 Mg^{2+} and Ca^{2+} 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 Ca^{2+} and Mg^{2+}. In heart, the predominant forms of this latter group of compounds include homocarnosine and anserine with a total concentration of ≈20 mmol/L.[15] The affinities of Ca^{2+} and Mg^{2+} for these histidyl dipeptides are lower than that of ATP, and together they constitute the bulk of the diffusible Ca^{2+} buffers. One feature of diffusible Ca^{2+} buffers is their ability to increase the apparent diffusion coefficient of Ca^{2+} 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 Ca^{2+} buffering (see below).

Buffer Kinetics

The importance of the different kinetics of the major buffers is illustrated in Figure 2Bi. The amount of Ca^{2+} bound to the regulatory site of TnC lags slightly behind free $[Ca^{2+}]$. The lag is much greater for the slower buffers (here the Mg^{2+} 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-

Circulation. 2019;139:2358–2371. DOI: 10.1161/CIRCULATIONAHA.118.039329

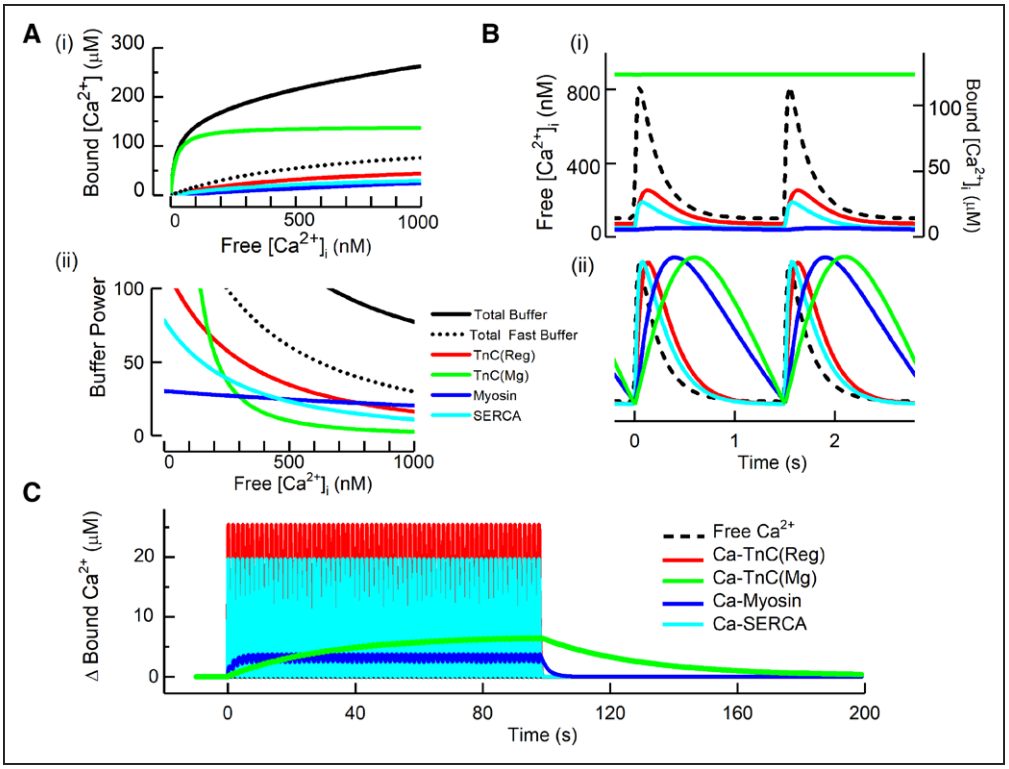

Figure 2. Properties of cellular cardiac Ca^{2+} buffers.
Ai, Steady-state Ca^{2+} buffering showing the dependence of bound on free Ca^{2+}. The colored curves show the contributions of the 2 Ca^{2+} binding sites (regulatory [Reg] and Mg) of TnC, myosin, and SERCA. The solid black line shows the total, 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 $[Ca^{2+}]_i$. **B**, Time course of change of bound $[Ca^{2+}]$ in response to systolic Ca^{2+} transients applied at 1.5 Hz. **i**, Absolute levels of $[Ca^{2+}]$. The dashed line shows free $[Ca^{2+}]$ and the colored traces show the concentration of the Ca^{2+}-bound form of the various ligands. **ii**, Normalized concentrations to emphasize kinetics. **C**, The change of bound Ca^{2+} in response to a series of Ca^{2+} transients (not shown) applied at 1.5 Hz. SERCA indicates sarcoplasmic reticulum Ca^{2+} ATPase pump; and TnC, troponin C.

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

MEASUREMENT OF Ca^{2+} BUFFERING

As discussed above, buffering depends on the summed effects of a variety of Ca^{2+} 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 al[31] studied isolated cardiac myofilaments and calculated that about 22 μmol of Ca^{2+} per kg heart is required to produce 50% maximum contraction. This was accompanied by a rise of free Ca^{2+} of ≈1.4 μmol/L, indicating that the myofilaments alone can bind >90% of the total Ca^{2+}. A subsequent approach, using cardiac homogenates, found that to raise free $[Ca^{2+}]$ to 1 μmol/L required 72 μmol/kg total Ca^{2+}.[32] Hove-Madsen and Bers[33] 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 Ca^{2+} current[20,34] under conditions in which Ca^{2+} removal mechanisms were inhibited (see Figure 3A and 3B). Berlin et al[34] compared Ca^{2+} entry with the rise of $[Ca^{2+}]_i$, 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 Ca^{2+} through sodium calcium exchange (NCX) with $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ which then decays as NCX removes Ca^{2+} from the cell. Integrating the NCX current gives a measure of the change of total Ca^{2+} concentration

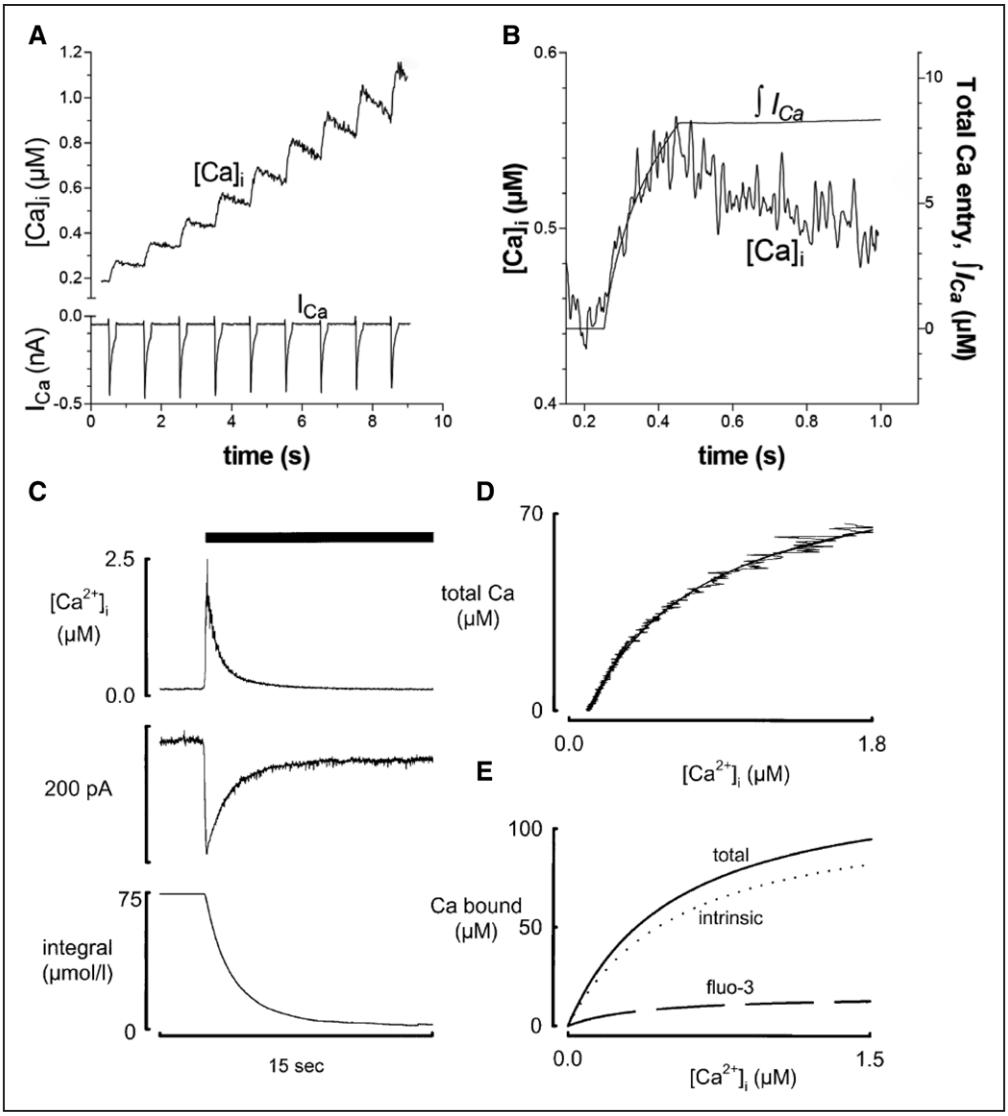

Figure 3. Measurement of buffering in intact cells.
A, Comparison of the effects of Ca^{2+} influx through the L-type Ca^{2+} channel (**bottom**) with the resulting increase of $[Ca^{2+}]_i$. **B**, Relationship between the integral of the L-type Ca^{2+} current and the change of $[Ca^{2+}]_i$. Ca^{2+} removal by SR, mitochondria, NCX, and PMCA were inhibited with thapsigargin, a mitochondrial uncoupler, Na-free solution and elevated external Ca^{2+} concentration, respectively. Figure reproduced from Bers[35] from an original article[34] with permission. Copyright © 2001, Kluwer Academic. **C**, Determination of Ca^{2+} buffering from the caffeine-evoked release of Ca^{2+} from the SR. Traces show (from top to bottom): $[Ca^{2+}]_i$, NCX current, integral of current. Caffeine (10 mmol/L) was applied as shown by the bar. **D**, Relationship between total Ca^{2+} (estimated from the integral of NCX current) and $[Ca^{2+}]_i$. **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 Ca^{2+} ATPase; and SR, sarcoplasmic reticulum.

that is compared continuously with the change of free $[Ca^{2+}]_i$ to characterize buffers[36] (Figure 3C through 3E). The caffeine response typically decays with a time constant of 1 to 2 s[38] 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 cytoplasm[36] 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 Ca^{2+} 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 Ca^{2+} TRANSIENT

Alterations of buffering power affect the systolic Ca^{2+} transient and thence contraction. Incorporation of Ca-sensitive indicators has the side effect of increasing Ca^{2+} 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 Ca^{2+} transient because, the higher the buffer power, the smaller the change of free $[Ca^{2+}]$ resulting from a given rate of Ca^{2+} pumping.[43] Adding exogenous buffer also decreases the rate of spontaneous beating of sinoatrial node cells,

presumably by decreasing the changes of $[Ca^{2+}]_i$ 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 Ca^{2+} transient, slower buffers produce a biphasic decay. The initial, fast phase reflects the time taken for cytoplasmic Ca^{2+} to bind to the buffer with the slower phase depending on the kinetics of Ca^{2+} removal from the cytoplasm.[43]

It should be noted that, in the steady state, averaged over the cardiac cycle, Ca^{2+} efflux must equal influx. This efflux is determined by $[Ca^{2+}]_i$. If one assumes that Ca^{2+} efflux is proportional to $[Ca^{2+}]_i$, then, in the steady state, the decrease of amplitude of the Ca^{2+} 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 $[Ca^{2+}]_i$ is unaffected.[46,47] Increasing stimulation rate will load cytoplasmic Ca^{2+} buffers (Figure 2C). An interruption of beating will result in this extra Ca^{2+} 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 Ca^{2+} content in the steady state? Experimental studies have found that adding exogenous cytoplasmic buffers decreases SR Ca^{2+}.[43,48] One explanation is that SERCA activity depends in a cooperative manner on $[Ca^{2+}]_i$,[49] whereas NCX has a linear dependence.[50] The decreased amplitude of the systolic Ca^{2+} transient may therefore decrease SERCA activity more than NCX, leading to a net loss of SR Ca^{2+}. Further studies are required to see if the decrease of SR content with increased buffering is a general phenomenon.

FACTORS THAT ALTER Ca^{2+} BUFFERING

Diastolic $[Ca^{2+}]_i$

For a simple buffer, total ($[Ca_T]$) and free ($[Ca^{2+}]$) are related by:

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

where B_{max} is the total buffer concentration and K_d is the concentration of Ca^{2+} at which 50% of the buffer has Ca^{2+} 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 Ca^{2+} 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 $[Ca^{2+}]_i \approx 100$ nmol/L, the Mg^{2+} 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 $[Ca^{2+}]$ and decreases as $[Ca^{2+}]$ increases. When $[Ca^{2+}]=K_d$ 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 $[Ca^{2+}]_i$, the larger will be the increase of $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$, an increase of 60 μmol/L total $[Ca^{2+}]$ produces a larger increase of free $[Ca^{2+}]$ when applied from a higher diastolic $[Ca^{2+}]_i$ than from a lower. This is clear in the simulated transients of the upper graph of Figure 4D. An increase of diastolic $[Ca^{2+}]_i$ of only 100 nmol/L (from 50 to 150) increases systolic $[Ca^{2+}]_i$ by 500 nmol/L. Therefore, an increase of diastolic $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ has also been suggested to account for a rapid initial rate of decay of the Ca^{2+} transient.[54]

This consequence of changes of diastolic $[Ca^{2+}]_i$ will add to the inotropic effects of manoeuvers such as the addition of cardiac glycosides[55] or β-adrenergic stimulation[56] that can increase diastolic $[Ca^{2+}]_i$. It is also a possible explanation for changes of the amplitude of the Ca^{2+} transient and force under the many conditions where there are no measurements of diastolic $[Ca^{2+}]_i$. Testing this will require obtaining and comparing absolute measurements of $[Ca^{2+}]_i$ between cells or tissues from different animals or patients. There is a dearth of such measurements in the literature[57] because it is much easier to measure changes of fluorescence of a Ca^{2+}-sensitive indicator than absolute levels of $[Ca^{2+}]_i$. Properly calibrated measurements, ideally using ratiometric 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 $[Ca^{2+}]_i$ produced by a given increase of total cytoplasmic Ca^{2+}, contributing to the inotropic effects of increased rate.[52] This effect is analogous to that discussed above for elevated diastolic $[Ca^{2+}]_i$, but the slow kinetics result in a memory so that, following a change of rate, the effects on systolic $[Ca^{2+}]_i$ and thence on the action potential duration may

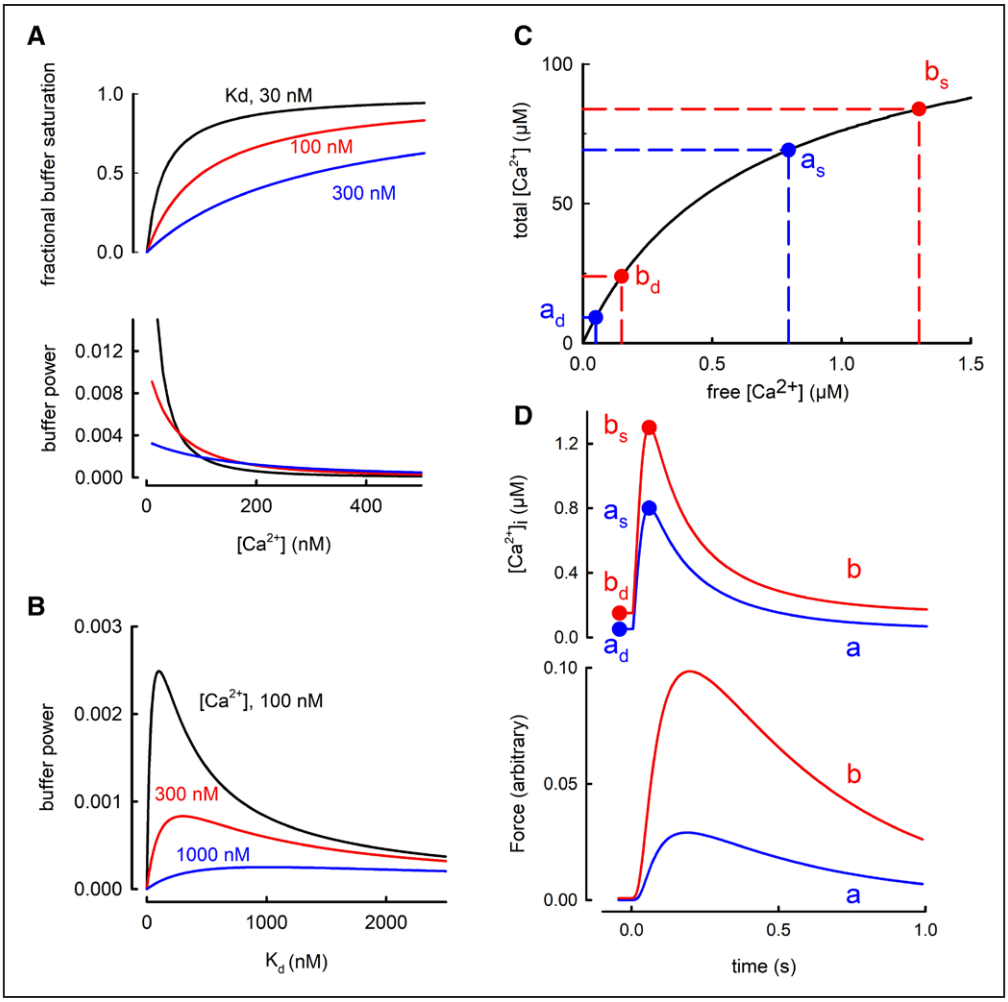

Figure 4. Effects of $[Ca^{2+}]_i$ and buffer K_d on buffer power.
A, Effects of $[Ca^{2+}]$. The top graph shows fractional saturation of a single buffer as a function of $[Ca^{2+}]$. Curves are shown for 3 different values of K_d. The lower graph shows calculated buffer power (change of total Ca^{2+}/change of $[Ca^{2+}]_i$) as a function of $[Ca^{2+}]$ for these values of K_d. Colors correspond to those above. **B**, Dependence of buffer power on K_d at the 3 values of $[Ca^{2+}]$ indicated. **C**, The effects of a small increase of diastolic $[Ca^{2+}]_i$ on the change of systolic $[Ca^{2+}]_i$ produced by the addition of a fixed amount of total $[Ca^{2+}]$. The buffer curve (fast buffers only) shows 2 levels of diastolic $[Ca^{2+}]_i$ 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 $[Ca^{2+}]$ resulting in a larger increase of systolic $[Ca^{2+}]_i$ in b compared with a. **D**, Simulation of the effect of adding and removing 60 μmol/L total Ca^{2+} with kinetics designed to represent a Ca^{2+} transient. Labels correspond to points in **C**. **Upper**, simulated Ca^{2+} transients; **Lower**, predicted force responses calculated using a published model.[53]

outlast those of diastolic $[Ca^{2+}]_i$. 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 $[Ca^{2+}]_i$, buffer power is greater the lower the value of K_d because this results in stronger Ca^{2+} binding. In contrast, at higher $[Ca^{2+}]_i$, the lower the K_d, the less the buffer power as the buffers become saturated (see[23] 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 $[Ca^{2+}]_i$ but decrease it at peak systolic ones.

One issue that has received no attention is whether Ca^{2+} buffering is the same in all cells in the ventricle. Given the regional differences of expression of other proteins including pumps[59] and channels,[60] heterogeneity of buffering would not be surprising. Likewise, possible variations of Ca^{2+} 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 Ca^{2+} buffers, TnC and SERCA, are regulated by the phosphorylation of troponin I and phospholamban, respectively, resulting in increased affinity

of Ca^{2+} for SERCA[61] and decreased affinity of Ca^{2+} 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 Ca^{2+} 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 Ca^{2+} affinity on buffer power will depend on the range of $[Ca^{2+}]_i$ under investigation.

Effects of Changes of pH on Buffering

Many Ca^{2+} buffers can bind protons as an alternative to Ca^{2+} ions. Direct measurements have shown that acidification decreases Ca^{2+} binding to troponin.[21] Therefore, acidification will decrease the affinity for Ca^{2+} with a decrease of Ca^{2+} 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 Ca^{2+} buffering.[63] We suggest that this may occur because, although a decrease of Ca^{2+} 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 Ca^{2+} 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 Ca^{2+} is pumped out of the cell. It may result from the inhibition of Ca^{2+} 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 Ca^{2+} BUFFERING

Atrial Buffering, Fibrillation, and Failure

The total concentration of Ca^{2+} 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 expression in the atrium than in the ventricle. Changes in Ca^{2+} 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 Ca^{2+} affinity of the buffers.[66] This decreased both the amplitude and rate of decay of the systolic Ca^{2+} transient. Atrial myocytes from many species, including rabbits and cats, have few or no t-tubules ([67] for review) and the systolic Ca^{2+} transient begins at the periphery of the cell and then propagates toward the center.[68,69] Increasing Ca^{2+} 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 al[71] 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 Ca^{2+} waves, thereby contributing to atrial fibrillation.[76] More work is required on changes of atrial Ca^{2+} 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.

Ca^{2+} Buffering and Heart Failure

Ca^{2+} buffering is unaffected by pacing-induced heart failure in both dogs[77] and sheep.[78] In contrast, in samples from human ventricle, the Ca^{2+} sensitivity of contraction was increased in dilated cardiomyopathy, possibly because of the decreased phosphorylation of troponin I.[79] Increased Ca^{2+} sensitivity was also found in canine dilated cardiomyopathy[80] and mouse infarct models.[81,82] As mentioned earlier, changes of Ca^{2+} sensitivity of contraction do not necessarily indicate altered Ca^{2+} binding and buffering; direct measurements of Ca^{2+} 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 Ca^{2+} buffering

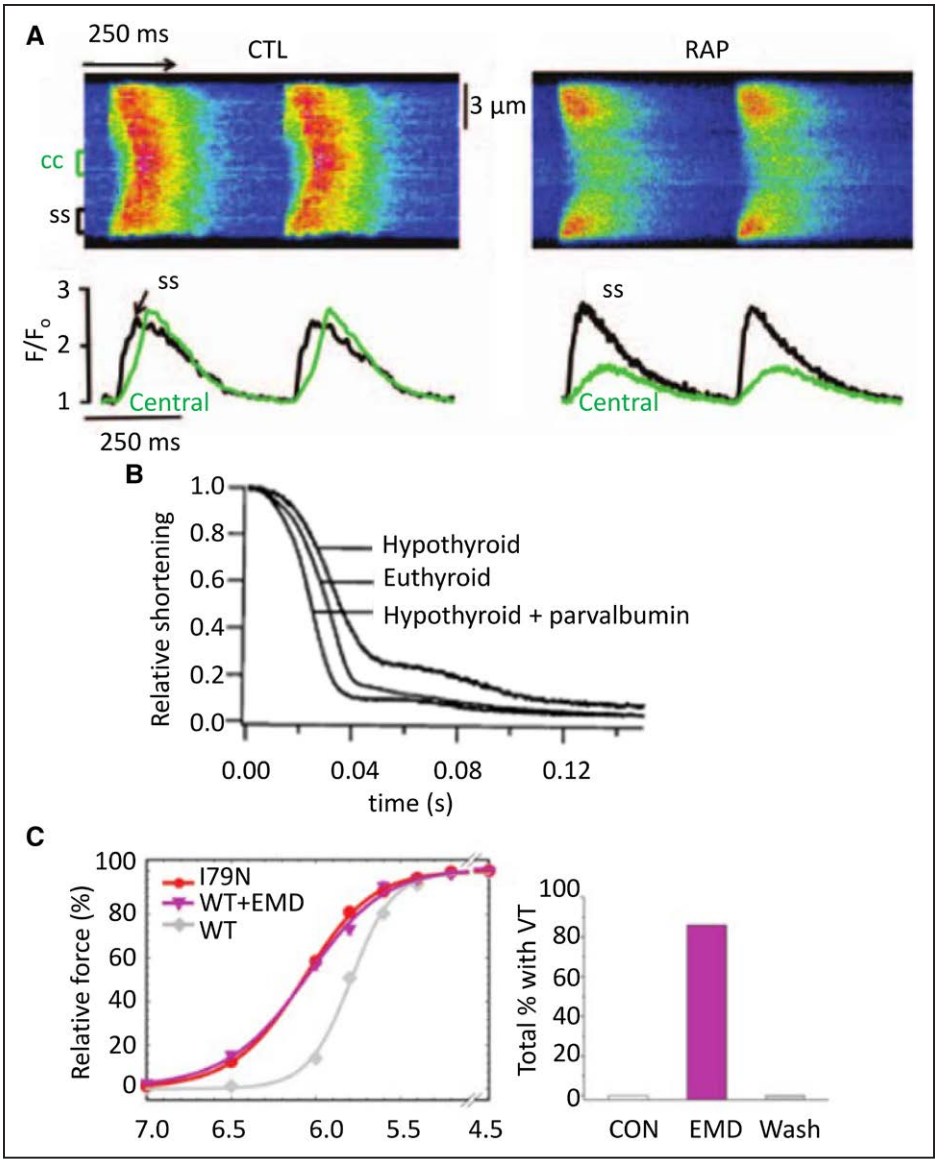

Figure 5. Effects of altered buffer power.
A, Abolition of centripetal propagation of the Ca^{2+} transient by rapid atrial pacing (RAP). Upper traces are linescan images. Lower records show $[Ca^{2+}]_i$ measured at surface of cell (black) and in center (green). **Left**, Records from control. **Right**, 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 Ca^{2+} 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 $[Ca^{2+}]_i$, 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 Ca^{2+} 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 troponin[85] 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 Ca^{2+} 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 $[Ca^{2+}]_i$ and, thence, of buffering power on the amplitude of the calcium transient. A major problem here is the paucity

of measurements of diastolic $[Ca^{2+}]_i$. It is essential that studies on heart failure ask the simple question: how accurately has diastolic $[Ca^{2+}]_i$ been measured and can it be excluded that changes (for example, between animals or in disease) account for the observed changes of systolic $[Ca^{2+}]_i$?

Work from Metzger and colleagues has demonstrated that changes of Ca^{2+} 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 Ca^{2+} buffers slow both the rise and fall of $[Ca^{2+}]_i$ and decrease the amplitude of the Ca^{2+} transient, and, instead, they advocated the use of parvalbumin, a skeletal muscle Ca^{2+} buffer. This has the important property that it binds Ca^{2+} slowly because Mg^{2+} has to dissociate first and, therefore, there is little attenuation of the peak Ca^{2+} transient. It will bind Ca^{2+} during diastole thereby improving diastolic performance. Incorporation of α-parvalbumin was shown to accelerate the decay of $[Ca^{2+}]_i$ with no effect on peak $[Ca^{2+}]_i$ and (see Figure 5B) also reversed the slowing of relaxation produced by experimental hypothyroidism[72] and in the Dahl salt-sensitive rat model of diastolic dysfunction.[87] Subsequent work has turned to altering the structure and thence the relative Ca^{2+} and Mg^{2+} 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 Mg^{2+} site of TnC and myosin.

Ca^{2+} 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 al[22] showed that mutations causing hypertrophic cardiomyopathy increased the binding affinity of Ca^{2+} to myofilaments (as assessed with a fluorescent troponin) and presumably therefore Ca^{2+} buffering. They proposed that alterations of buffering might lead to pathological changes of the Ca^{2+} 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 Ca^{2+} transient observed.[23,90] In addition, myofilament Ca^{2+} sensitization with EMD 57033 mimicked the effects of troponin T mutations on Ca^{2+} buffering and the Ca^{2+} transient.[23] A recent study used adenovirus to infect isolated myocytes with troponin or tropomyosin mutations and, again, found an increase of diastolic $[Ca^{2+}]_i$.[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 Ca^{2+} current. Although no data are available, it seems unlikely that ranolazine would affect Ca^{2+} buffering. It may therefore be that some of the effects of thin filament mutations are directly attributable to Ca^{2+} buffering, and others are a secondary consequence of the resulting heart failure, possibly attributable to decreased SERCA.

As mentioned in an earlier section, the Mg^{2+} sites on troponin are important contributors to buffering at low $[Ca^{2+}]_i$. It is therefore interesting that one of the mutations associated with FHC (D145E) greatly decreases the affinity of Ca^{2+} 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 Mg^{2+} TnC sites will actually increase Ca^{2+} buffering power in the systolic range of $[Ca^{2+}]_i$.

Ca^{2+} 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 Ca^{2+} (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 Ca^{2+} 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 Ca^{2+} 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 Ca^{2+} 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 Ca^{2+} transient was larger than control and this effect was more prominent in troponin T mutations that sensitize to activation by Ca^{2+}.[23] This effect was attributed to a higher cell Ca^{2+} content in the mutant during stimulation, with the excess Ca^{2+} 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 $[Ca^{2+}]_i$ and consequent decrease of buffer power may also increase the rise of $[Ca^{2+}]_i$ 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 Ca^{2+} sensitivity have been reported following myocardial infarction[81] where manoeuvers that decrease Ca^{2+} sensitivity were found to abolish ventricular tachycardia following pauses of stimulation. As reviewed above, an increase of Ca^{2+} buffering is proarrhythmogenic. It has also been shown, however, that addition of the buffer EGTA can prevent the propagation of arrhythmogenic Ca^{2+} waves,[95] and therefore the net effect may be more complicated.

The induction of alternans of the action potential duration by increased Ca^{2+} buffering attributable to thin filament mutations[73] may be a consequence of the slowed decay of the Ca^{2+} 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 Ca^{2+} transient because of lower SERCA expression.[59] In contrast, a modeling study has predicted that increasing cytoplasmic Ca^{2+} buffering should decrease the probability of alternans occurring by decreasing the probability that Ca^{2+} released from the SR induces further Ca^{2+} 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 alternans[48] and may be related to the experimental observation that increasing cytoplasmic Ca^{2+} buffering decreases the frequency of propagating Ca^{2+} waves[97] and makes Ca^{2+} sparks terminate earlier.[98] Further work is clearly required in understanding the relationship between Ca^{2+} buffering and alternans.

Why Do Cells Have Ca^{2+} Buffers?

A high level of Ca^{2+} buffering is not unique to cardiac myocytes. For example, ≈99% of the Ca^{2+} entering chromaffin cells binds to cytoplasmic buffers.[99] The presence of Ca^{2+} buffers means that much larger movements of total Ca^{2+} are required to produce a given change of $[Ca^{2+}]_i$. Given that calcium movements 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 Ca^{2+} as a second messenger requires high concentrations of Ca^{2+} binding proteins, for example, to activate contraction. (2) A high Ca^{2+} buffering may stabilize Ca^{2+} signaling by stopping an abnormal increase of $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ means that the buffer power is much lower in systole than diastole. This may help Ca^{2+} 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 6×10^{16} ions per liter corresponding to a mean distance between ions of 0.25 μm. Soeller and Cannell[8] have modeled Ca^{2+} 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 Ca^{2+} ions. At 10 μmol/L, there will be between 0.7 and 2.8 ions. This would make it impossible to control $[Ca^{2+}]_i$ in a stable manner because a single Ca^{2+} transported into or out of the space would result in an enormous fractional change of $[Ca^{2+}]_i$. 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 Ca^{2+} 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 Ca^{2+} 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 $[Ca^{2+}]_i$, contribute to alterations of calcium handling and contractility. The limited human data reviewed above and extrapolated from animal models argue that changes of Ca^{2+} 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.

Circulation. 2019;139:2358–2371. DOI: 10.1161/CIRCULATIONAHA.118.039329

ARTICLE INFORMATION

Correspondence

Godfrey L. Smith, PhD, Rm 415A, West Medical Building, University of Glasgow, Glasgow G12 8QQ, UK; or David A. Eisner, DPhil, 3.18, Core Technology Facility, University of Manchester, 46 Grafton St, Manchester M13 9NT, UK. Email godfrey.smith@glasgow.ac.uk or eisner@manchester.ac.uk

Affiliations

Institute of Cardiovascular and Medical Sciences, College of Medical, Veterinary, and Life Sciences, University of Glasgow, UK (G.L.S.). Unit of Cardiac Physiology, Division of Cardiovascular Sciences, University of Manchester, UK (D.A.E.).

Acknowledgments

We are indebted to F. Burton and S. Wray for comments on an earlier version of the manuscript and to Q. Lachaud for the design of Figure 1.

Sources of Funding

This work from is supported by grants from the British Heart Foundation (PG/17/12/32847 to Dr Smith and CH/2000004/12801 to Dr Eisner).

Disclosures

None.

REFERENCES

1. Bers DM. Calcium cycling and signaling in cardiac myocytes. *Annu Rev Physiol.* 2008;70:23–49. doi: 10.1146/annurev.physiol.70.113006.100455
2. Eisner DA, Caldwell JL, Kistamás K, Trafford AW. Calcium and excitation-contraction coupling in the heart. *Circ Res.* 2017;121:181–195. doi: 10.1161/CIRCRESAHA.117.310230
3. Fabiato A. Calcium-induced release of calcium from the cardiac sarcoplasmic reticulum. *Am J Physiol.* 1983;245:C1–14. doi: 10.1152/ajpcell.1983.245.1.C1
4. Williams RJ. The evolution of calcium biochemistry. *Biochim Biophys Acta.* 2006;1763:1139–1146. doi: 10.1016/j.bbamcr.2006.08.042
5. Carafoli E, Krebs J. Why calcium? How calcium became the best communicator. *J Biol Chem.* 2016;291:20849–20857. doi: 10.1074/jbc.R116.735894
6. Kretsinger RH, Nockolds CE. Carp muscle calcium-binding protein. II. Structure determination and general description. *J Biol Chem.* 1973;248:3313–3326.
7. Saucerman JJ, Bers DM. Calmodulin mediates differential sensitivity of CaMKII and calcineurin to local Ca2+ in cardiac myocytes. *Biophys J.* 2008;95:4597–4612. doi: 10.1529/biophysj.108.128728
8. Soeller C, Cannell MB. Numerical simulation of local calcium movements during L-type calcium channel gating in the cardiac diad. *Biophys J.* 1997;73:97–111. doi: 10.1016/S0006-3495(97)78051-2
9. Robertson SP, Johnson JD, Potter JD. The time-course of Ca2+ exchange with calmodulin, troponin, parvalbumin, and myosin in response to transient increases in Ca2+. *Biophys J.* 1981;34:559–569. doi: 10.1016/S0006-3495(81)84868-0
10. Gao WD, Backx PH, Azan-Backx M, Marban E. Myofilament Ca2+ sensitivity in intact versus skinned rat ventricular muscle. *Circ Res.* 1994;74:408–415.
11. Shannon TR, Ginsburg KS, Bers DM. Reverse mode of the sarcoplasmic reticulum calcium pump and load-dependent cytosolic calcium decline in voltage-clamped cardiac ventricular myocytes. *Biophys J.* 2000;78:322–333. doi: 10.1016/S0006-3495(00)76595–7
12. Post JA, Langer GA. Sarcolemmal calcium binding sites in heart: I. Molecular origin in "gas-dissected" sarcolemma. *J Membr Biol.* 1992;129:49–57.
13. Pan BS, Solaro RJ. Calcium-binding properties of troponin C in detergent-skinned heart muscle fibers. *J Biol Chem.* 1987;262:7839–7849.
14. Picht E, Zima AV, Shannon TR, Duncan AM, Blatter LA, Bers DM. Dynamic calcium movement inside cardiac sarcoplasmic reticulum during release. *Circ Res.* 2011;108:847–856. doi: 10.1161/CIRCRESAHA.111.240234
15. Swietach P, Youm JB, Saegusa N, Leem CH, Spitzer KW, Vaughan-Jones RD. Coupled Ca2+/H+ transport by cytoplasmic buffers regulates local Ca2+ and H+ ion signaling. *Proc Natl Acad Sci U S A.* 2013;110:E2064–E2073. doi: 10.1073/pnas.1222433110
16. Baran EJ. Metal complexes of carnosine. *Biochemistry (Mosc).* 2000;65:789–797.
17. Naraghi M. T-jump study of calcium binding kinetics of calcium chelators. *Cell Calcium.* 1997;22:255–268. doi: 10.1016/S0143-4160(97)90064–6
18. Smith PD, Liesegang GW, Berger RL, Czerlinski G, Podolsky RJ. A stopped-flow investigation of calcium ion binding by ethylene glycol bis(beta-aminoethyl ether)-N,N'-tetraacetic acid. *Anal Biochem.* 1984;143:188–195.
19. Kawasaki H, Kretsinger RH. Calcium-binding proteins. 1: EF-hands. *Protein Profile.* 1994;1:343–517.
20. Sipido KR, Wier WG. Flux of Ca2+ across the sarcoplasmic reticulum of guinea-pig cardiac cells during excitation-contraction coupling. *J Physiol.* 1991;435:605–630.
21. Blanchard EM, Solaro RJ. Inhibition of the activation and troponin calcium binding of dog cardiac myofibrils by acidic pH. *Circ Res.* 1984;55:382–391.
22. Robinson P, Griffiths PJ, Watkins H, Redwood CS. Dilated and hypertrophic cardiomyopathy mutations in troponin and alpha-tropomyosin have opposing effects on the calcium affinity of cardiac thin filaments. *Circ Res.* 2007;101:1266–1273. doi: 10.1161/CIRCRESAHA.107.156380
23. Schober T, Huke S, Venkataraman R, Gryshchenko O, Kryshtal D, Hwang HS, Baudenbacher FJ, Knollmann BC. Myofilament Ca sensitization increases cytosolic Ca binding affinity, alters intracellular Ca homeostasis, and causes pause-dependent Ca-triggered arrhythmia. *Circ Res.* 2012;111:170–179. doi: 10.1161/CIRCRESAHA.112.270041
24. Robinson P, Liu X, Sparrow A, Patel S, Zhang YH, Casadei B, Watkins H, Redwood C. Hypertrophic cardiomyopathy mutations increase myofilament Ca2+ buffering, alter intracellular Ca2+ handling, and stimulate Ca2+-dependent signaling. *J Biol Chem.* 2018;293:10487–10499. doi: 10.1074/jbc.RA118.002081
25. Siddiqui JK, Tikunova SB, Walton SD, Liu B, Meyer M, de Tombe PP, Neilson N, Kekenes-Huskey PM, Salhi HE, Janssen PM, Biesiadecki BJ, Davis JP. Myofilament calcium sensitivity: consequences of the effective concentration of troponin I. *Front Physiol.* 2016;7:632. doi: 10.3389/fphys.2016.00632
26. Powers FM, Solaro RJ. Caffeine alters cardiac myofilament activity and regulation independently of Ca2+ binding to troponin C. *Am J Physiol.* 1995;268(6 Pt 1):C1348–C1353. doi: 10.1152/ajpcell.1995.268.6.C1348
27. Negele JC, Dotson DG, Liu W, Sweeney HL, Putkey JA. Mutation of the high affinity calcium binding sites in cardiac troponin C. *J Biol Chem.* 1992;267:825–831.
28. Higgins ER, Cannell MB, Sneyd J. A buffering SERCA pump in models of calcium dynamics. *Biophys J.* 2006;91:151–163. doi: 10.1529/biophysj.105.075747
29. Shannon TR, Wang F, Puglisi J, Weber C, Bers DM. A mathematical treatment of integrated Ca dynamics within the ventricular myocyte. *Biophys J.* 2004;87:3351–3371. doi: 10.1529/biophysj.104.047449
30. Michailova A, DelPrincipe F, Egger M, Niggli E. Spatiotemporal features of Ca2+ buffering and diffusion in atrial cardiac myocytes with inhibited sarcoplasmic reticulum. *Biophys J.* 2002;83:3134–3151. doi: 10.1016/S0006-3495(02)75317-4
31. Solaro RJ, Wise RM, Shiner JS, Briggs FN. Calcium requirements for cardiac myofibrillar activation. *Circ Res.* 1974;34:525–530.
32. Pierce GN, Philipson KD, Langer GA. Passive calcium-buffering capacity of a rabbit ventricular homogenate preparation. *Am J Physiol.* 1985;249(3 pt 1):C248–C255. doi: 10.1152/ajpcell.1985.249.3.C248
33. Hove-Madsen L, Bers DM. Passive Ca buffering and SR Ca uptake in permeabilized rabbit ventricular myocytes. *Am J Physiol.* 1993;264(3 pt 1):C677–C686. doi: 10.1152/ajpcell.1993.264.3.C677
34. Berlin JR, Bassani JW, Bers DM. Intrinsic cytosolic calcium buffering properties of single rat cardiac myocytes. *Biophys J.* 1994;67:1775–1787. doi: 10.1016/S0006-3495(94)80652-6
35. Bers DM. *Excitation-Contraction Coupling and Cardiac Contractile Force*. 2nd ed. Dordrecht, The Netherlands: Kluwer Academic; 2001.
36. Trafford AW, Díaz ME, Eisner DA. A novel, rapid and reversible method to measure Ca buffering and time-course of total sarcoplasmic reticulum Ca content in cardiac ventricular myocytes. *Pflugers Arch.* 1999;437:501–503. doi: 10.1007/s004240050808
37. Kuratomi S, Matsuoka S, Sarai N, Powell T, Noma A. Involvement of Ca2+ buffering and Na+/Ca2+ exchange in the positive staircase of contraction in guinea-pig ventricular myocytes. *Pflugers Arch.* 2003;446:347–355. doi: 10.1007/s00424-003-1023-1
38. Negretti N, O'Neill SC, Eisner DA. The relative contributions of different intracellular and sarcolemmal systems to relaxation in rat ventricular myocytes. *Cardiovasc Res.* 1993;27:1826–1830. doi: 10.1093/cvr/27.10.1826

39. Trafford AW, Díaz ME, Sibbring GC, Eisner DA. Modulation of CICR has no maintained effect on systolic Ca2+: simultaneous measurements of sarcoplasmic reticulum and sarcolemmal Ca2+ fluxes in rat ventricular myocytes. *J Physiol.* 2000;522(pt 2):259–270.
40. Briston SJ, Dibb KM, Solaro RJ, Eisner DA, Trafford AW. Balanced changes in Ca buffering by SERCA and troponin contribute to Ca handling during β-adrenergic stimulation in cardiac myocytes. *Cardiovasc Res.* 2014;104:347–354. doi: 10.1093/cvr/cvu201
41. Steenbergen C, Murphy E, Levy L, London RE. Elevation in cytosolic free calcium concentration early in myocardial ischemia in perfused rat heart. *Circ Res.* 1987;60:700–707.
42. Harding DP, Smith GA, Metcalfe JC, Morris PG, Kirschenlohr HL. Resting and end-diastolic [Ca2+]i measurements in the Langendorff-perfused ferret heart loaded with a 19F NMR indicator. *Magn Reson Med.* 1993;29:605–615.
43. Díaz ME, Trafford AW, Eisner DA. The effects of exogenous calcium buffers on the systolic calcium transient in rat ventricular myocytes. *Biophys J.* 2001;80:1915–1925. doi: 10.1016/S0006-3495(01)76161-9
44. Yaniv Y, Stern MD, Lakatta EG, Maltsev VA. Mechanisms of beat-to-beat regulation of cardiac pacemaker cell function by Ca^{2+} cycling dynamics. *Biophys J.* 2013;105:1551–1561. doi: 10.1016/j.bpj.2013.08.024
45. Kaestner L, Scholz A, Tian Q, Ruppenthal S, Tabellion W, Wiesen K, Katus HA, Müller OJ, Kotlikoff MI, Lipp P. Genetically encoded Ca2+ indicators in cardiac myocytes. *Circ Res.* 2014;114:1623–1639. doi: 10.1161/CIRCRESAHA.114.303475
46. Neher E. Usefulness and limitations of linear approximations to the understanding of Ca++ signals. *Cell Calcium.* 1998;24:345–357. doi: 10.1016/S0143-4160(98)90058-6
47. Sankaranarayanan R, Kistamás K, Greensmith DJ, Venetucci LA, Eisner DA. Systolic [Ca2+]i regulates diastolic levels in rat ventricular myocytes. *J Physiol.* 2017;595:5545–5555. doi: 10.1113/JP274366
48. Kornyeyev D, Reyes M, Escobar AL. Luminal Ca(2+) content regulates intracellular Ca(2+) release in subepicardial myocytes of intact beating mouse hearts: effect of exogenous buffers. *Am J Physiol Heart Circ Physiol.* 2010;298:H2138–H2153. doi: 10.1152/ajpheart.00885.2009
49. Lytton J, Westlin M, Burk SE, Shull GE, MacLennan DH. Functional comparisons between isoforms of the sarcoplasmic or endoplasmic reticulum family of calcium pumps. *J Biol Chem.* 1992;267:14483–14489.
50. Barcenas-Ruiz L, Beuckelmann DJ, Wier WG. Sodium-calcium exchange in heart: membrane currents and changes in [Ca2+]i. *Science.* 1987;238:1720–1722.
51. MacQuaide N, Dempster J, Smith GL. Measurement and modeling of Ca2+ waves in isolated rabbit ventricular cardiomyocytes. *Biophys J.* 2007;93:2581–2595. doi: 10.1529/biophysj.106.102293
52. Gattoni S, Røe ÅT, Frisk M, Louch WE, Niederer SA, Smith NP. The calcium-frequency response in the rat ventricular myocyte: an experimental and modelling study. *J Physiol.* 2016;594:4193–4224. doi: 10.1113/JP272011
53. Hunter PJ, McCulloch AD, ter Keurs HE. Modelling the mechanical properties of cardiac muscle. *Prog Biophys Mol Biol.* 1998;69:289–331.
54. Díaz ME, Trafford AW, Eisner DA. The role of intracellular Ca buffers in determining the shape of the systolic Ca transient in cardiac ventricular myocytes. *Pflugers Arch.* 2001;442:96–100. doi: 10.1007/s004240000509
55. Wier WG, Hess P. Excitation-contraction coupling in cardiac Purkinje fibers. Effects of cardiotonic steroids on the intracellular [Ca2+] transient, membrane potential, and contraction. *J Gen Physiol.* 1984;83:395–415. doi: 10.1016/S0079-6107(98)00013-3
56. Sankaranarayanan R, Li Y, Greensmith DJ, Eisner DA, Venetucci L. Biphasic decay of the Ca transient results from increased sarcoplasmic reticulum Ca leak. *J Physiol.* 2016;594:611–623. doi: 10.1113/JP271473
57. Eisner DA. Ups and downs of calcium in the heart. *J Physiol.* 2018;596:19–30. doi: 10.1113/JP275130
58. Eisner DA, Dibb KM, Trafford AW. The mechanism and significance of the slow changes of ventricular action potential duration following a change of heart rate. *Exp Physiol.* 2009;94:520–528. doi: 10.1113/expphysiol.2008.044008
59. Laurita KR, Katra R, Wible B, Wan X, Koo MH. Transmural heterogeneity of calcium handling in canine. *Circ Res.* 2003;92:668–675. doi: 10.1161/01.RES.0000062468.25308.27
60. Soltysinska E, Olesen SP, Christ T, Wettwer E, Varró A, Grunnet M, Jespersen T. Transmural expression of ion channels and transporters in human nondiseased and end-stage failing hearts. *Pflugers Arch.* 2009;459:11–23. doi: 10.1007/s00424-009-0718-3
61. Kirchberber MA, Tada M, Katz AM. Phospholamban: a regulatory protein of the cardiac sarcoplasmic reticulum. *Recent Adv Stud Cardiac Struct Metab.* 1975;5:103–115.
62. Robertson SP, Johnson JD, Holroyde MJ, Kranias EG, Potter JD, Solaro RJ. The effect of troponin I phosphorylation on the Ca2+-binding properties of the Ca2+-regulatory site of bovine cardiac troponin. *J Biol Chem.* 1982;257:260–263.
63. Choi HS, Trafford AW, Orchard CH, Eisner DA. The effect of acidosis on systolic Ca2+ and sarcoplasmic reticulum calcium content in isolated rat ventricular myocytes. *J Physiol.* 2000;529(pt 3):661–668. doi: 10.1111/j.1469-7793.2000.00661.x
64. Boyman L, Hagen BM, Giladi M, Hiller R, Lederer WJ, Khananshvili D. Proton-sensing Ca2+ binding domains regulate the cardiac Na+/Ca2+ exchanger. *J Biol Chem.* 2011;286:28811–28820. doi: 10.1074/jbc.M110.214106
65. Walden AP, Dibb KM, Trafford AW. Differences in intracellular calcium homeostasis between atrial and ventricular myocytes. *J Mol Cell Cardiol.* 2009;46:463–473. doi: 10.1016/j.yjmcc.2008.11.003
66. Clarke JD, Caldwell JL, Pearman CM, Eisner DA, Trafford AW, Dibb KM. Increased Ca buffering underpins remodelling of Ca2+ handling in old sheep atrial myocytes. *J Physiol.* 2017;595:6263–6279. doi: 10.1113/JP274053
67. Trafford AW, Clarke JD, Richards MA, Eisner DA, Dibb KM. Calcium signalling microdomains and the t-tubular system in atrial myocytes: potential roles in cardiac disease and arrhythmias. *Cardiovasc Res.* 2013;98:192–203. doi: 10.1093/cvr/cvt018
68. Blatter LA, Kockskämper J, Sheehan KA, Zima AV, Hüser J, Lipsius SL. Local calcium gradients during excitation-contraction coupling and alternans in atrial myocytes. *J Physiol.* 2003;546(pt 1):19–31. doi: 10.1113/jphysiol.2002.025239
69. Bootman MD, Higazi DR, Coombes S, Roderick HL. Calcium signalling during excitation-contraction coupling in mammalian atrial myocytes. *J Cell Sci.* 2006;119(Pt 19):3915–3925. doi: 10.1242/jcs.03223
70. Sheehan KA, Blatter LA. Regulation of junctional and non-junctional sarcoplasmic reticulum calcium release in excitation-contraction coupling in cat atrial myocytes. *J Physiol.* 2003;546(pt 1):119–135. doi: 10.1113/jphysiol.2002.026963
71. Greiser M, Kerfant BG, Williams GS, Voigt N, Harks E, Dibb KM, Giese A, Meszaros J, Verheule S, Ravens U, Allessie MA, Gammie JS, van der Velden J, Lederer WJ, Dobrev D, Schotten U. Tachycardia-induced silencing of subcellular Ca2+ signaling in atrial myocytes. *J Clin Invest.* 2014;124:4759–4772. doi: 10.1172/JCI70102
72. Wahr PA, Michele DE, Metzger JM. Parvalbumin gene transfer corrects diastolic dysfunction in diseased cardiac myocytes. *Proc Natl Acad Sci USA.* 1999;96:11982–11985. doi: 10.1073/pnas.96.21.11982
73. Baudenbacher F, Schober T, Pinto JR, Sidorov VY, Hilliard F, Solaro RJ, Potter JD, Knollmann BC. Myofilament Ca2+ sensitization causes susceptibility to cardiac arrhythmia in mice. *J Clin Invest.* 2008;118:3893–3903. doi: 10.1172/JCI36642
74. Clarke JD, Caldwell JL, Horn MA, Bode EF, Richards MA, Hall MC, Graham HK, Briston SJ, Greensmith DJ, Eisner DA, Dibb KM, Trafford AW. Perturbed atrial calcium handling in an ovine model of heart failure: potential roles for reductions in the L-type calcium current. *J Mol Cell Cardiol.* 2015;79:169–179. doi: 10.1016/j.yjmcc.2014.11.017
75. Dibb KM, Clarke JD, Horn MA, Richards MA, Graham HK, Eisner DA, Trafford AW. Characterization of an extensive transverse tubular network in sheep atrial myocytes and its depletion in heart failure. *Circ Heart Fail.* 2009;2:482–489. doi: 10.1161/CIRCHEARTFAILURE.109.852228
76. Macquaide N, Tuan HT, Hotta J, Sempels W, Lenaerts I, Holemans P, Hofkens J, Jafri MS, Willems R, Sipido KR. Ryanodine receptor cluster fragmentation and redistribution in persistent atrial fibrillation enhance calcium release. *Cardiovasc Res.* 2015;108:387–398. doi: 10.1093/cvr/cvv231
77. Hobai IA, O'Rourke B. Enhanced Ca(2+)-activated Na(+)-Ca(2+) exchange activity in canine pacing-induced heart failure. *Circ Res.* 2000;87:690–698.
78. Briston SJ, Caldwell JL, Horn MA, Clarke JD, Richards MA, Greensmith DJ, Graham HK, Hall MC, Eisner DA, Dibb KM, Trafford AW. Impaired β-adrenergic responsiveness accentuates dysfunctional excitation-contraction coupling in an ovine model of tachypacing-induced heart failure. *J Physiol.* 2011;589(pt 6):1367–1382. doi: 10.1113/jphysiol.2010.203984
79. Wolff MR, Buck SH, Stoker SW, Greaser ML, Mentzer RM. Myofibrillar calcium sensitivity of isometric tension is increased in human dilated cardiomyopathies: role of altered beta-adrenergically mediated protein phosphorylation. *J Clin Invest.* 1996;98:167–176. doi: 10.1172/JCI118762
80. Wolff MR, Whitesell LF, Moss RL. Calcium sensitivity of isometric tension is increased in canine experimental heart failure. *Circ Res.* 1995;76:781–789.
81. Venkataraman R, Baldo MP, Hwang HS, Veltri T, Pinto JR, Baudenbacher FJ, Knollmann BC. Myofilament calcium de-sensitization and contractile uncoupling prevent pause-triggered ventricular tachycardia in mouse hearts with chronic myocardial infarction. *J Mol Cell Cardiol.* 2013;60:8–15. doi: 10.1016/j.yjmcc.2013.03.022

82. de Waard MC, van der Velden J, Bito V, Ozdemir S, Biesmans L, Boontje NM, Dekkers DH, Schoonderwoerd K, Schuurbiers HC, de Crom R, Stienen GJ, Sipido KR, Lamers JM, Duncker DJ. Early exercise training normalizes myofilament function and attenuates left ventricular pump dysfunction in mice with a large myocardial infarction. *Circ Res.* 2007;100:1079–1088. doi: 10.1161/01.RES.0000262655.16373.37
83. Belin RJ, Sumandea MP, Kobayashi T, Walker LA, Rundell VL, Urboniene D, Yuzhakova M, Ruch SH, Geenen DL, Solaro RJ, de Tombe PP. Left ventricular myofilament dysfunction in rat experimental hypertrophy and congestive heart failure. *Am J Physiol Heart Circ Physiol.* 2006;291:H2344–H2353. doi: 10.1152/ajpheart.00541.2006
84. Solaro RJ, van der Velden J. Why does troponin I have so many phosphorylation sites? Fact and fancy. *J Mol Cell Cardiol.* 2010;48:810–816. doi: 10.1016/j.yjmcc.2010.02.014
85. Marjot J, Kaier TE, Martin ED, Reji SS, Copeland ON, Iqbal M, Goodson B, Hamren S, Harding SE, Marber MS. Quantifying the release of biomarkers of myocardial necrosis from cardiac myocytes and intact myocardium. *Clinical Chem*. 2017;63:990–996. doi: 10.1373/clinchem.2016.264648
86. Meyer M, Schillinger W, Pieske B, Holubarsch C, Heilmann C, Posival H, Kuwajima G, Mikoshiba K, Just H, Hasenfuss G. Alterations of sarcoplasmic reticulum proteins in failing human dilated cardiomyopathy. *Circulation.* 1995;92:778–784. doi: 10.1161/01.CIR.92.4.778
87. Rodenbaugh DW, Wang W, Davis J, Edwards T, Potter JD, Metzger JM. Parvalbumin isoforms differentially accelerate cardiac myocyte relaxation kinetics in an animal model of diastolic dysfunction. *Am J Physiol Heart Circ Physiol.* 2007;293:H1705–H1713. doi: 10.1152/ajpheart.00232.2007
88. Zhang J, Shettigar V, Kindell D, Liu X, Lopez J, Yerrimuni V, Davis G and Davis J. Engineering parvalbumin for the heart: optimizing the Mg^{2}+ binding properties of rat β-parvalbumin. *Front Physiol*. 2011;2:77. doi: 10.3389/fphys.2011.00077
89. Asp ML, Sjaastad FV, Siddiqui JK, Davis JP, Metzger JM. Effects of modified parvalbumin EF-hand motifs on cardiac myocyte contractile function. *Biophys J.* 2016;110:2094–2105. doi: 10.1016/j.bpj.2016.03.037
90. Li Y, Zhang L, Jean-Charles PY, Nan C, Chen G, Tian J, Jin JP, Gelb IJ, Huang X. Dose-dependent diastolic dysfunction and early death in a mouse model with cardiac troponin mutations. *J Mol Cell Cardiol.* 2013;62:227–236. doi: 10.1016/j.yjmcc.2013.06.007
91. Coppini R, Mazzoni L, Ferrantini C, Gentile F, Pioner JM, Laurino A, Santini L, Bargelli V, Rotellini M, Bartolucci G, Crocini C, Sacconi L, Tesi C, Belardinelli L, Tardiff J, Mugelli A, Olivotto I, Cerbai E, Poggesi C. Ranolazine prevents phenotype development in a mouse model of hypertrophic cardiomyopathy. *Circ Heart Fail*. 2017;10:e003565. doi: 10.1161/CIRCHEARTFAILURE.116.003565
92. Swindle N, Tikunova SB. Hypertrophic cardiomyopathy-linked mutation D145E drastically alters calcium binding by the C-domain of cardiac troponin C. *Biochemistry.* 2010;49:4813–4820. doi: 10.1021/bi100400h
93. Maron BJ. Sudden death in young athletes. *N Engl J Med.* 2003;349:1064–1075. doi: 10.1056/NEJMra022783
94. Wang L, Kryshtal DO, Kim K, Parikh S, Cadar AG, Bersell KR, He H, Pinto JR, Knollmann BC. myofilament calcium-buffering dependent action potential triangulation in human-induced pluripotent stem cell model of hypertrophic cardiomyopathy. *J Am Coll Cardiol.* 2017;70:2600–2602. doi: 10.1016/j.jacc.2017.09.033
95. MacQuaide N, Ramay HR, Sobie EA, Smith GL. Differential sensitivity of Ca^{2}+ wave and Ca^{2}+ spark events to ruthenium red in isolated permeabilised rabbit cardiomyocytes. *J Physiol.* 2010;588(pt 23):4731–4742. doi: 10.1113/jphysiol.2010.193375
96. Nivala M, Qu Z. Calcium alternans in a couplon network model of ventricular myocytes: role of sarcoplasmic reticulum load. *Am J Physiol Heart Circ Physiol.* 2012;303:H341–H352. doi: 10.1152/ajpheart.00302.2012
97. Nivala M, Ko CY, Nivala M, Weiss JN, Qu Z. Criticality in intracellular calcium signaling in cardiac myocytes. *Biophys J.* 2012;102:2433–2442. doi: 10.1016/j.bpj.2012.05.001
98. Bovo E, Mazurek SR, Fill M, Zima AV. Cytosolic Ca^{2+} buffering determines the intra-SR Ca^{2+} concentration at which cardiac Ca^{2+} sparks terminate. *Cell Calcium.* 2015;58:246–253. doi: 10.1016/j.ceca.2015.06.002
99. Neher E, Augustine GJ. Calcium gradients and buffers in bovine chromaffin cells. *J Physiol.* 1992;450:273–301.
100. Gibbs CL, Loiselle DS, Wendt IR. Activation heat in rabbit cardiac muscle. *J Physiol.* 1988;395:115–130.