Ca\(^{2+}\) buffering in the heart: Ca\(^{2+}\) binding to and activation of cardiac myofibrils

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The measurement of cardiac Ca\(^{2+}\) transients using spectroscopic Ca\(^{2+}\) indicators is significantly affected by the buffering properties of the indicators. The aim of the present study was to construct a model of cardiac Ca\(^{2+}\) buffering that satisfied the kinetic constraints imposed by the maximum attainable rates of cardiac contraction and relaxation on the Ca\(^{2+}\) dissociation rate constants and which would account for the observed effects of \(^{19}\)F-NMR indicators on the cardiac Ca\(^{2+}\) transient in the Langendorff-perfused ferret heart. It is generally assumed that the Ca\(^{2+}\) dependency of myofibril activation in cardiac myocytes is mediated by a single Ca\(^{2+}\)-binding site on troponin C. A model based on 1:1 Ca\(^{2+}\) binding to the myofilaments, however, was unable to reproduce our experimental data, but a model in which we assumed ATP-dependent co-operative Ca\(^{2+}\) binding to the myofilaments was able to reproduce these data. This model was used to calculate the concentration and dissociation constant of the ATP-independent myofilament Ca\(^{2+}\) binding, giving 58 and 2.0 \(\mu\)M respectively. In addition to reproducing our experimental data on the concentration of free Ca\(^{2+}\) ions in the cytoplasm ([Ca\(^{2+}\)]), the resulting Ca\(^{2+}\) and ATP affinities given by fitting of the model also provided good predictions of the Ca\(^{2+}\) dependency of the myofibrillar ATPase activity measured under in vitro conditions. Solutions to the model also indicate that the Ca\(^{2+}\) mobilized during each beat remains unchanged in the presence of the additional buffering load from Ca\(^{2+}\) indicators.

The new model was used to estimate the extent of perturbation of the Ca\(^{2+}\) transient caused by different concentrations of indicators. As little as 10 \(\mu\)M of a Ca\(^{2+}\) indicator with a dissociation constant of 200 \(\mu\)M will cause a 20% reduction in peak-systolic [Ca\(^{2+}\)] and 30 \(\mu\)M will cause approx. 50% reduction in the peak-systolic [Ca\(^{2+}\)], in a heart paced at 1.0 Hz.

Key words: actomyosin-ATPase, Ca\(^{2+}\) metabolism, Ca\(^{2+}\)-transient, kinetics, troponin.

INTRODUCTION

The concentration of free Ca\(^{2+}\) ions in the cytoplasm ([Ca\(^{2+}\)]\(_i\)) is critical for the control of cardiac contraction (reviewed in [1]). The normal cardiac [Ca\(^{2+}\)]\(_i\) rises from a diastolic value of approx. 160 nM to a systolic value of approx. 2700 nM [2]. These values are determined by the amount of Ca\(^{2+}\) entering and leaving the cytosol during each cardiac cycle and the kinetic properties of the cytoplasmic Ca\(^{2+}\) buffers. Most information on cardiac [Ca\(^{2+}\)]\(_i\) comes from studies using spectroscopic Ca\(^{2+}\) indicators (e.g. [2,3] and references therein). These indicators can accurately report [Ca\(^{2+}\)]\(_i\) in biological tissues, but they also chelate Ca\(^{2+}\) and therefore act as buffers [4]. Exogenous indicators, by adding to the buffering capacity, will therefore report a [Ca\(^{2+}\)]\(_i\) that may be significantly different from the unperturbed [Ca\(^{2+}\)]\(_i\).

Endogenous Ca\(^{2+}\) buffers in the heart may be divided into three groups based on their affinities. High-affinity buffers (association constant \(>3 \times 10^7\) M\(^{-1}\), e.g. calmodulin [5]) will be more than 80% bound in diastole and will not contribute significantly to buffering of the cardiac Ca\(^{2+}\) transient. Conversely, buffers with low affinities (association constant \(<10^6\) M\(^{-1}\)) will not bind an appreciable amount of Ca\(^{2+}\) during the transient. The major contribution to cytosolic Ca\(^{2+}\) buffering during the Ca\(^{2+}\) transient will therefore be from those species with affinities in the range \(10^4–10^6\) M\(^{-1}\). Total cardiac Ca\(^{2+}\) buffering (i.e. including high-, intermediate- and low-affinity buffers) has been estimated from equilibrium dialysis studies to be in the range 250–300 \(\mu\)M [6,7]. More recently, Bers and colleagues have estimated ‘fast’ Ca\(^{2+}\) buffering (i.e. buffering that occurs within the time scale of the Ca\(^{2+}\) transient), assuming that Ca\(^{2+}\) buffering could be represented by a single aggregate Michaelis–Menten-type species, to have a maximum binding capacity of 123 \(\mu\)M with an apparent dissociation constant of 0.96 \(\mu\)M [8].

The major species thought to buffer Ca\(^{2+}\) during the cardiac Ca\(^{2+}\) transient is the troponin C component of the myofibrils [9,10]. In vitro studies suggest that troponin C and intact myofibrils have only one Ca\(^{2+}\)-binding site per troponin C with an affinity in the physiologically relevant range [11,12]. However, recent biochemical studies have shown that in the presence of ATP, the myofibrils bind Ca\(^{2+}\) co-operatively, but still only one per troponin C, with an apparent \(K_d\) in the \(\mu\)M range [13]. In contrast with the Ca\(^{2+}\) dependency of both Ca\(^{2+}\) binding and the ATPase activity of myofibrils, the ATP dependency of the ATPase has a Hill coefficient of one. In the present study we have constructed a new model of cardiac Ca\(^{2+}\) buffering based on ATP-dependent co-operative Ca\(^{2+}\) binding to the myofilaments and used the model to calculate the affinities and concentrations of endogenous Ca\(^{2+}\) buffers and the extent of perturbation of Ca\(^{2+}\) transients that will be caused by loading Ca\(^{2+}\) indicators.

MATERIALS AND METHODS

\(^{19}\)F-NMR database

The buffering of [Ca\(^{2+}\)]\(_i\) by Ca\(^{2+}\) indicators is dependent on the concentration of indicator loaded and the binding affinity of the indicator. In a previous study we found that the intracellular con-

Abbreviations used: ATP refers generally to the total adenosine triphosphate in the presence of at least 1 mM Mg\(^{2+}\), i.e. near saturated [MgATP\(^{2+}\)]; [Ca\(^{2+}\)]\(_i\), concentration of free Ca\(^{2+}\) ions in the cytoplasm; PCR, phosphorecreatine; SOS, sum of squares.

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concentration of 3H-labelled 1,2-bis-[2-bis(carboxymethyl)amino-5-fluorophenoxo]ethane (5FBAPTA) in hearts with a signal-to-noise ratio of 120. It was assumed that all indicators were present at a concentration of 100 nM. All indicators were loaded until the S/N ratio was approx. 10:1. We have therefore assumed that all indicators were present at a concentration of 120 nM [2]. The differences in the buffering of the Ca2+-transient between the different indicators will therefore predominantly reflect differences in their Ca2+-affinities. The experimental determinations of diastolic and systolic [Ca2+] in the perfused ferret heart, for indicators with Ki values ranging from 46 to 2950 nM, are summarized in Table 1.

The data sets used in this study were acquired under two limiting sets of conditions. The [Ca2+] measurements in the intact heart were obtained under isometric conditions. The ATPase measurements cited were obtained in free solution under isotonic, zero-force, conditions.

In the isometric state there is no external work performed; the ATPase continues only until sufficient cross-bridges are formed to stiffen the fibres and to generate the ‘internal’ shortening of the fibres. The experimental determinations of diastolic and systolic [Ca2+] in the perfused ferret heart, for indicators with Ki values ranging from 46 to 2950 nM, are summarized in Table 1.

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The ATPase runs until the maximal shortening is reached. The number of steps of shortening obtained per ATP consumed depends on the work done by the muscle, which is minimal when the force is zero. The ATPase data have been normalized as fractional. The removal of proportionality factors by the normalization process data has allowed the use of the same equilibrium equations to generate the model predicted [Ca2+] and [ATP] dependencies of the ATPase (see the Results section).

Construction of the new kinetic model of Ca2+ buffering in the intact heart is based in part qualitatively on data for the [Ca2+] dependence of the myofibrillar ATPase activity shown in Figure 7 of Holroyde et al. [11] and quantitatively on data for the [ATP] dependence of the myofibrillar ATPase activity shown in Figure 2 of Krause and Jacobus [15] (both data sets used with permission of the authors).

**Computer methods**

Solutions to the models were found by an iterative alteration of the unknown variables until the sums of squares (SOSs) of the difference between the experimentally measured values and those predicted by the model were minimized, using the Microsoft Excel 98 Solver (Microsoft Corporation, Seattle, WA, U.S.A.). The only constraint imposed was that all parameters be positive. The functions to be minimized were highly non-linear in terms of the known variables (concentrations and affinities); therefore the quadratic extrapolation method was used to estimate the unknown variables for each one-dimensional search. Central differencing was used to refine the solutions obtained. To improve the rate of approach to solution, the conjugate gradient search method was used (Microsoft Excel-5 Users Guide, p. 574). Automatic scaling in the Excel Solver was unable to function optimally when the parameters differed by many orders of magnitude and so in this case manual scaling of the variables, by the use of log values, was also required. Where combined SOSs were used, a weighting was applied so that the contribution to the solution process was the same for both sets of data.

An empirical function for the variation of fraction of indicator bound to Ca2+ in diastole ([FCa]/[Fl]o) with indicator affinity (logK) was found by fitting those data (Table 1) to an arbitrary two-term sigmoidal function, [FCa]/[Fl]o = 1/(1 + e−bK + cK), shown as a solid line in Figure 5. This function was then used to generate the model-derived increase in fraction bound of indicator on moving to systole as a continuous function of logK (see Figure 3) and the expected fraction bound (dotted line in Figure 5). The empirical function was used solely for presentation of the results and was not used in the fitting process.

**RESULTS**

**Inadequacies in simple models of the myofibril**

Hill coefficient of Ca2+ activation of the myofibrils in vitro is greater than one

The data for [Ca2+] dependence of the myofibrillar ATPase (replotted from Figure 7 of Holroyde et al. [11]) are shown in Figure 1. Also shown in Figure 1 are the best fits to the data for activation by 1:1 and highly co-operative 2:1 binding of Ca2+ to the myofibrils (dotted line and solid line respectively) based on the binding schemes shown in Scheme 1 for regulation by Ca2+, in which M represents the myofibrils and K1 and K2 are the affinity constants for the first and second ions bound.

The 1:1 binding provides a very poor fit to the results; this indicates that the process exhibits co-operativity. The 2:1 model provides a much better fit, although at high [Ca2+], where the data will be most accurate, a model with more than two Ca2+ binding would fit better. Therefore the myofibrillar ATPase data of Holroyde et al. [11] indicate that Ca2+ binding to the myofibrils in vitro strongly co-operate so the concentration of singly ligated myofibrillar protein will be small.

**Limitations of the 2:1 Ca2+ binding model: the affinities of individual Ca2+-binding steps**

The pCa for half-activation of the myofibrils is 6.1 (see Figure 1). In general, from product data only, it is not possible to determine...
the affinities of the individual binding steps. However, it is possible to impose limits on the affinities of the individual steps.

If we consider the two-step binding scheme, then the equation for the fraction of maximal rate of reaction for the ATPase would be:

\[ v = V_{\text{max}} \frac{K_1 K_2 [\text{Ca}^{2+}]^2}{1 + K_1 [\text{Ca}^{2+}] + K_1 K_2 [\text{Ca}^{2+}]^2} \]

where \( K_1 \) and \( K_2 \) represent the affinities of the two \( \text{Ca}^{2+} \)-binding steps.

If the value for the affinity of one of the binding steps (e.g. \( K_1 \)) is set, then, from any data set the corresponding value for the affinity of the other binding step (\( K_2 \)) can be found by least-squares fitting. The values for \( K_2 \) found for the ATPase data over a wide range of values of \( K_1 \) \((10^{-5} - 10^{6} \text{ M}^{-1})\) and the corresponding deviations (SOSs) from ideal fit are shown in Figure 2. At values of \( K_1 > 10^4 \text{ M}^{-1} \) the SOS increased significantly. This suggests that if \( \text{Ca}^{2+} \) binding to the myofibrils follows the simple two-step \( \text{Ca}^{2+} \) binding scheme, as described by eqn. (1), then for this model \( K_2 \) must be less than \( 10^4 \text{ M}^{-1} \) and therefore \( K_1 \) must be more than \( 10^5 \text{ M}^{-1} \) (see Figure 2). This is not consistent with the \( \text{Ca}^{2+} \) affinity of troponin C \((10^{4-6} \text{ M}^{-1})\); e.g. [12]).

A further deficiency of this model becomes clear when account is taken of the rate at which relaxation must occur for the cardiac cycle to function at the observed maximum rate. The cardiac cycle can operate at rates up to 3–10 Hz (depending on species). For a rate of 5 Hz (the maximum observed for ferret [16]) this corresponds to systolic and diastolic durations of less than 100 ms each. To allow full relaxation of the myofibrils, more than 95% of the second \( \text{Ca}^{2+} \) must have dissociated from the myofibrils, i.e. the dissociation rate must be more than 50 s\(^{-1}\). If we assume that the \( \text{Ca}^{2+} \) on-rate for the myofibrils is maximal, i.e. limited by diffusion at approx. \( 5 \times 10^{10} \text{ M}^{-1} \cdot \text{s}^{-1} \) [17], then the upper limit for the affinity of the second \( \text{Ca}^{2+} \) binding step (\( K_2 \)) would be \( 5 \times 10^5/50 \), i.e. \( 10^7 \text{ M}^{-1} \). In practice the high protein concentration makes the diffusion rate in the cytosol considerably less than that in water. Therefore the data fitting, shown in Figure 2, indicating that \( K_2 \) must be considerably greater than \( 10^7 \text{ M}^{-1} \), is not compatible with the kinetic constraints of the rates of contraction and relaxation of the myofibrils. This means that \( \text{Ca}^{2+} \) binding to the myofibrils cannot be explained by the simple two-step binding process described in eqn. (1). By analogous reasoning, \( \text{Ca}^{2+} \) binding to the myofibrils cannot be explained by a third-order or higher-order allosteric regulated binding scheme. Therefore, given that \( \text{Ca}^{2+} \) binding to the myofibrils is co-operative (see Figure 1), \( \text{Ca}^{2+} \) binding to the myofibrils must be modulated by other factors.

Limitations of the 2:1 \( \text{Ca}^{2+} \) binding model: the Hill coefficient for ATP activation of the myofibrillar ATPase is unity

It has previously been proposed that co-operativity between cross-bridges could explain the high Hill coefficient of the [\( \text{Ca}^{2+} \)] activation of the ATPase. This would be equivalent to \( M \) in Scheme (1) being two separate enzyme units both binding \( \text{Ca}^{2+} \) and ATP and working in concert. This mechanism is inconsistent with the lack of co-operativity seen for the [ATP] dependency of the ATPase (see the Discussion section). In addition, the limits to \( \text{Ca}^{2+} \) affinity set by the maximum cardiac rate would also apply for this model.

Resolution of the simple model problems

Modulation of \( \text{Ca}^{2+} \) binding by ATP

The obvious candidate for modulation of \( \text{Ca}^{2+} \) binding to the myofibrils is ATP, the substrate for the ATPase. Taylor and Weeds [18] have shown that the rate-limiting step of the

![Scheme 1](image)

![Figure 1](image)  

**Figure 1** \( \text{Ca}^{2+} \) dependence of myofibrillar ATPase

Data points (○) are reproduced, with permission, from Holroyde et al. [11]. Lines represent best fits to the data for 1:1 \( \text{Ca}^{2+} \) (dotted line) and 2:1 \( \text{Ca}^{2+} \) (solid line) binding schemes as described in Scheme (1) (see the text). The \([\text{Ca}^{2+}]\) at which the activity of the ATPase is half maximal is \(10^{-4.5} \text{ M}\).

![Figure 2](image)  

**Figure 2** Limits to the affinities for individual binding steps for the 2:1 \( \text{Ca}^{2+} \)-binding scheme

The effect of fixing the value for the first \( \text{Ca}^{2+} \) affinity, \( K_1 \), in the range \(10^3 - 10^9 \text{ M}^{-1}\) for eqn. (2), in which the ATPase is proportional to \([\text{MCA}_2]\), is shown. Right ordinate (broken line), the value found for \( K_1 \) corresponding to each set value of \( K_1 \). Left ordinate (solid line), the SOS for the best fit to the data for values of \( K_1 \). Clearly at values of \( \log K_1 > 5 \) the SOS starts to increase significantly. A very high value for \( K_1 \) would ensures that the first \( \text{Ca}^{2+} \) could not dissociate, which would make the process effectively first-order, already shown to give a very poor fit to the data (Figure 1).
myofibrillar ATPase was the rate of ADP dissociation, which has a rate constant of 65 s$^{-1}$. The similarity of the rate of ATP hydrolysis with the minimum constraint imposed by the rate of relaxation (50 s$^{-1}$; see above) suggests that it may be the rate of ATP hydrolysis that limits the rate of relaxation. If binding a second Ca$^{2+}$ depended on ATP, e.g. if the affinity were high in the presence of bound ATP (low dissociation rate) but low in the absence of bound ATP (fast dissociation rate), then the effective Ca$^{2+}$ dissociation rate would approach that of the ADP dissociation rate, i.e. 65 s$^{-1}$. In effect, the affinity of the Ca$^{2+}$ binding could be much greater than 10$^{6}$ M$^{-1}$ in the presence of bound ATP, yet still have an effective dissociation rate of approx. 50 s$^{-1}$.

Recent $^{45}$Ca-binding studies have shown that, in the presence of ATP, Ca$^{2+}$ binding to the myofibrils has increased co-operativity [13,19], which is consistent with ATP dependence of the Ca$^{2+}$ binding. However, it is not possible to determine from the data of [13] the exact degree of co-operativity in the presence of ATP because of the inaccuracies in determining the Hill coefficient of Ca$^{2+}$ binding and the possibility of diffusion-limited control of local ATP levels within myofibrils as seen during ATPase measurements. In addition to Ca$^{2+}$-binding being ATP dependent (see above), Arata et al. [20] have shown that, in the presence of low [Ca$^{2+}$], ATP inhibits contractility, indicating that there is substrate inhibition. This substrate inhibition suggests that there is a degree of ordered binding of the Ca$^{2+}$ and ATP to the myofibrils.

The simplest model that can explain the co-operative [Ca$^{2+}$] data of Holroyde et al. [11] and the ATP dependency of Ca$^{2+}$ binding shown by Morimoto and Ohtsuki [13] and Tobacman [19] is illustrated by steps $K_1$, $K_2$, and $K_3$ in Scheme 2. This we shall refer to as the Ca–ATP–Ca model.

Additional features incorporated into the new kinetic model of Ca$^{2+}$ binding

In addition to the basic scheme ($K_1-K_2-K_3$) illustrated in Scheme 2, we have incorporated additional features into the new kinetic model of Ca$^{2+}$ binding to the myofibrils based on the following observations.

(i) The apparent ATP affinity of the myofibrillar ATPase approaches a high limiting value when the [Ca$^{2+}$] is increased [15]. The ATP-induced Ca$^{2+}$-binding co-operativity [19] implies ATP binding before the second Ca$^{2+}$. The scheme allows a second Ca$^{2+}$ to bind before ATP ($K_t$ in Scheme 2), as well as after ATP ($K_s$). For this scheme the apparent ATP affinity at very high [Ca$^{2+}$] will be $K_tK_s/K_r$. Without the ATP-independent Ca$^{2+}$ binding, i.e. if $K_s$ were to be zero, the apparent affinity for ATP would increase indefinitely with [Ca$^{2+}$].

(ii) The myofibrils can bind ATP in the absence of Ca$^{2+}$ ($K_t$ in Scheme 2), termed the loose-bound ATP state [21]. The value of $K_t$ measured from the ATP-dependent changes in fluorescence of the isolated myofibrils, is approx. 10$^{3}$ M$^{-1}$ [18,21]. The substrate inhibition shown by Arata et al. [20] could arise from this binding.

Summary of the model of Ca$^{2+}$ binding

In summary, the studies of Holroyde et al. [11], Tobacman [19] and Morimoto and Ohtsuki [13] indicate that Ca$^{2+}$ binding to the myofibrils is co-operative and ATP dependent. A complete description of Ca$^{2+}$ binding to the myofibrils must also take into account the possibility that two Ca$^{2+}$ can bind without ATP [19] and that ATP can bind with or without one or both Ca$^{2+}$ [21] (Scheme 2).

Derivation of a new kinetic model of Ca$^{2+}$ buffering in the intact heart

Although the myofibrils represent the major source of Ca$^{2+}$ binding during the cardiac Ca$^{2+}$ transient, any model of Ca$^{2+}$ buffering in the intact heart must also consider the contributions of other endogenous buffers and any exogenous buffers (e.g. Ca$^{2+}$ indicators loaded into the heart). Furthermore, to calculate the buffering of Ca$^{2+}$ in the intact heart it is also necessary to know how much Ca$^{2+}$ enters the cytoplasm (from extracellular as well as intracellular stores) during each contraction.

Ca$^{2+}$ binding to the myofibrils

From the mass action equations applicable to Scheme 2 an expression can be derived for the total concentration of myofibrils, $[M]_T$:

$$[M]_T = [M]_0(1 + K_s[Ca^{2+}] + K_s[ATP] + K_tK_s[ATP][Ca^{2+}]) + K_rK_t[Ca^{2+}] + K_rK_sK_t[ATP][Ca^{2+}]^2$$

(2)

where $K_t$, $K_r$, $K_s$ and $K_r$ are the affinity constants for the myofibrillar Ca$^{2+}$ binding and the myofibrillar ATP binding (see Scheme 2), and $[M]_T$, [M] are the concentration of total myofilaments and free myofilaments.

Because we are studying Ca$^{2+}$ dependency, it is convenient to have [Ca$^{2+}$] as the sole variable and treat [ATP] as a parameter, so that the equation may be simplified to

$$[M]_T = M(\alpha + \beta[Ca^{2+}] + \gamma[Ca^{2+}]^2)$$

$$\alpha = 1 + K_s[ATP]$$

$$\beta = K_t + K_s[ATP]$$

(3)

$$\gamma = K_rK_t[ATP]$$
and hence we can obtain an expression for the fraction of the myofibrils activated as MCA\textsubscript{ATP}, denoted \(Q\), which for the case of the Ca–ATP–Ca model (Scheme 2) is:

\[
Q = \frac{K_a K_i [\text{ATP}][\text{Ca}^{2+}]^2}{\alpha + [\text{Ca}^{2+}] + \gamma [\text{Ca}^{2+}]^2}
\]  

(4)

\(\text{Ca}^{2+}\) binding to other endogenous \(\text{Ca}^{2+}\) buffers

Other non-myofibrillar \(\text{Ca}^{2+}\)-binding species, e.g. calmodulin and proteins on the cytoplasmic leaflet of both the sarcolemma and the sarcoplasmic reticulum membranes [9,10], may also contribute to \(\text{Ca}^{2+}\) buffering in the intact heart. If we assume that the additional endogenous \(\text{Ca}^{2+}\) buffers can be considered as a single species that binds one \(\text{Ca}^{2+}\) ion, then the following expression for the total \(\text{Ca}^{2+}\), \(\text{[Ca]}_T\), in the cytoplasm can be derived:

\[
[\text{Ca}]_T = [\text{Ca}] + [\text{M}]_T \left( \frac{(\beta[\text{Ca}] + 2\gamma[\text{Ca}^{2+}])}{(\alpha + [\text{Ca}] + \gamma[\text{Ca}^{2+}]^2)} \right) + [\text{E}]_T \frac{K_i [\text{Ca}^{2+}]}{1 + K_i [\text{Ca}^{2+}]} 
\]  

(5)

where \([\text{E}]_T\) is the total amount of other endogenous buffers attributed an affinity constant of \(K_i\).

As the 1:1 endogenous buffers would reduce the effective Hill coefficient of the cytosolic buffering, similar equations have also been derived for models where the additional endogenous \(\text{Ca}^{2+}\) buffers bind \(\text{Ca}^{2+}\) co-operatively (see the Discussion section).

\(\text{Ca}^{2+}\) binding to exogenous \(\text{Ca}^{2+}\) buffers (\(\text{Ca}^{2+}\) indicators)

To calculate \(\text{Ca}^{2+}\) buffering in the presence of \(\text{Ca}^{2+}\) indicators we measure the change in the fraction of indicator bound to \(\text{Ca}^{2+}\) between diastole and systole [2], \(\Delta[\text{FCa}\text{/}[\text{F}]_T\), i.e.:

\[
\Delta[\text{FCa}\text{/}[\text{F}]_T = \frac{[\text{FCa}]_S}{[\text{F}]_T} - \frac{[\text{FCa}]_D}{[\text{F}]_T}
\]  

(6)

where \([\text{FCa}]\) and \([\text{F}]_T\) are the concentrations of \(\text{Ca}^{2+}\) bound and total indicator. The subscripts S and D refer to systole and diastole.

\(\text{Ca}^{2+}\) entry into the cytoplasm during the \(\text{Ca}^{2+}\) transient

To calculate the buffering of [\(\text{Ca}^{2+}\)]i in the intact heart it is necessary to know how much \(\text{Ca}^{2+}\) enters the cytoplasm (\(\Delta[\text{Ca}]_T\)) as well as the change in [\(\text{Ca}^{2+}\)]i during each contraction. For the purposes of the model it is assumed that all sources of \(\text{Ca}^{2+}\) entry (from extracellular as well as intracellular sources) can be combined into a single \(\text{Ca}^{2+}\) source. As a first approximation we have assumed that the total \(\text{Ca}^{2+}\) mobilized during the cardiac cycle is independent of total (endogenous + exogenous) cytosolic \(\text{Ca}^{2+}\) buffering (see the Discussion section). \(\Delta[\text{Ca}]_T\) can then be calculated as the sum of the changes in [\(\text{Ca}^{2+}\)]i, \(\text{Ca}^{2+}\) bound to the myofibrils, \(\text{Ca}^{2+}\) bound to other endogenous buffers, and \(\text{Ca}^{2+}\) bound to the indicator, between diastole and systole:

\[
\Delta[\text{Ca}]_T = [\text{Ca}]_T - [\text{Ca}]_D + (\Sigma M[\text{Ca}]_S - \Sigma M[\text{Ca}]_D) + (\Sigma [\text{E}[\text{Ca}]]_S - \Sigma [\text{E}[\text{Ca}]]_D)) + \Delta[\text{FCa}]
\]  

(7)

The terms are used to indicate all species that are bound to either one or two \(\text{Ca}^{2+}\) with or without ATP (see Scheme 2).

Substitutions can be made to reduce this equation to a function of the known or measurable variables and parameters: [\(\text{Ca}^{2+}\)], [\(\text{ATP}\)] and \(K_i\), and the seven unknowns, namely \(\Delta[\text{Ca}]_T\), \([\text{M}]_T\), \([\text{E}]_T\), \(K_i\), \(K_x\), \(K_y\) and \(K_z\), which can be expressed as a function of the measured variable, the change in fraction of indicator bound to \(\text{Ca}^{2+}\), \(\Delta[\text{FCa}]_T/[\text{F}]_T\):

\[
\Delta[\text{FCa}]_T/[\text{F}]_T = \left( \frac{[\text{F}]_T}{[\text{F}]_T} \right) \left( \frac{[\text{FCa}]_D}{[\text{F}]_T} \right) - [\text{Ca}^{2+}] \left( \frac{1 + \frac{[\text{M}]_T + 2[\text{Ca}^{2+}]}{1 + \frac{[\text{Ca}]_D + [\text{Ca}]_D + \gamma [\text{Ca}^{2+}]^2}}}{1 + \frac{[\text{Ca}]_D + [\text{Ca}]_D + \gamma [\text{Ca}^{2+}]^2}} \right)
\]  

(8)

This equation therefore represents the new kinetic model of \(\text{Ca}^{2+}\) buffering in the intact heart that we have used to fit the NMR data for indicator \(K_x\)-dependent decline in [\(\text{Ca}^{2+}\)]i and thereby to estimate parameters for \(\text{Ca}^{2+}\) binding to the myofibrils and other endogenous \(\text{Ca}^{2+}\) buffers in the heart.

Constraining the kinetic model of \(\text{Ca}^{2+}\) buffering in the intact heart to fit the experimental data

To determine the parameters for \(\text{Ca}^{2+}\) buffering in the intact heart we constrained the model to fit two independent sets of results: first, the changes in the \(\text{Ca}^{2+}\)-transient following the loading of \(\text{19F}\)–NMR \(\text{Ca}^{2+}\) indicators in the Langendorff-perfused ferret heart [2] and second, the [\(\text{ATP}\)] dependence of the myofibril ATPase activity [15].

ATPase is treated as measured under equilibrium conditions

In the heart the ATP hydrolysis step is rendered essentially irreversible by the very low [\(\text{P}\)] maintained by the phosphate pumping of the mitochondria. The ATPase measurements are initial rates and may also be considered irreversible. In the derivation of the model the equilibrium equations were for the isometric condition where the total ATPase turnover is limited by steric constraints and hence time is available for equilibrium of the species of Scheme 2 to be reached.

For the isolated ATPase measurements the near equilibrium condition may not hold in the steady state. The rate-limiting step returns the myofibril, presumably with one or two \(\text{Ca}^{2+}\) bound, to the active pool. The prerequisite for the maximum possible rate of relaxation of the myofibril is that the dissociation rates of the \(\text{Ca}^{2+}\)-binding steps (\(K_i\) and \(K_x\)), and consequently any subsequent ATP binding, must be faster than 50 s\(^{-1}\). This minimum rate is close to the rate-limiting ADP dissociation rate. Therefore, for the Ca–ATP–Ca model, these steps will approach equilibrium in the steady state. Thus any appreciable displacement from equilibrium will result only from the relative rates of formation and dissociation of the MCA\textsubscript{ATP} species and the loss of this species by the rate-limiting ADP dissociation rate. Therefore, for the Ca–ATP–Ca model, these steps will approach equilibrium in the steady state. Thus any appreciable displacement from equilibrium will result only from the relative rates of formation and dissociation of the MCA\textsubscript{ATP} species and the loss of this species by the rate-limiting ADP dissociation rate. However, in the steady state the concentration of MCA\textsubscript{ATP} will be proportional to its rate of formation, i.e. the concentration of the precursor (MCA\textsubscript{ATP}) multiplied by the [\(\text{Ca}^{2+}\)] \(\times \) a constant. If normalized rates (fraction of maximal ATPase) are used then the constant disappears from the numerator giving eqn. (4). The rate-limiting step returns the myofibril to the pool equilibrating with \(\text{Ca}^{2+}\) and ATP-bound species and is included as MCA\textsubscript{ATP} in the sum of species making up the total myofibril concentration in
The changes observed (○) are shown. The line represents the best fit for the Ca–ATP–Ca model. Values for the systolic [Ca\(^{2+}\)] in the presence of 1,2-bis-[2-(1-carboxyethyl)(carboxymethyl)amino-4-fluorophenoxyl]ethane (‘MFBAPTA’ and 1,2-bis-[2-(1-carboxyethyl)(carboxymethyl)amino-4-fluorophenoxyl]ethane (‘DIME-FBAPTA’) (Δ) were allowed to vary because there were no experimental measurements of these values, and so they made no contribution to the sum-of-squares calculations. Values for the systolic [Ca\(^{2+}\)], for all indicators, however, were included in the extrapolation process to determine the unperturbed systolic and diastolic [Ca\(^{2+}\)], in each model solution. In all cases the values for unperturbed diastolic and systolic [Ca\(^{2+}\)], for the best fits of the models were in the range 150 ± 10 nM and 2670 ± 50 nM respectively. Both values are very similar to those obtained in the accompanying paper [2].

The best fit shown for the Ca–ATP–Ca model was obtained by simultaneously constraining the model to fit both the NMR data and the [ATP] dependence of the ATPase (see Figure 4). The fractional increases calculated are for the point measurements only. A continuous function for the increase between diastole and systole was derived as detailed in computational methods and Figure 5. Outside the range of indicator affinities used the continuous function is unreliable; the resultant curve is therefore plotted as dotted line in these regions. However, as expected the increase tends to zero each side of the data range available.

Figure 3 Increase between diastole and systole in the fraction of 19F indicator that is Ca\(^{2+}\) bound

The derivation of normalized ATPase rates.

To test the validity of using the equilibrium equations for the ATPase in the model, we have multiplied by a factor the value of [M\(\text{Ca}_4\text{ATP}\)] predicted by assuming equilibrium. The closeness of fitting the combined NMR and [ATP] data was critically dependent on this factor, which approached unity when allowed to vary during the solution process (more than 0.95, dependent on stringency).

Limits for the second ATP-independent Ca\(^{2+}\)-binding strength

In the physiological range of [Ca\(^{2+}\)], the Ca\(^{2+}\) binding to myofibrils measured in the absence of ATP appears to be one per troponin unit and is clearly not positively co-operative [13], which would seem to be at odds with the model derived above, unless \(K_s < K_i\) and not titrated in the range of [Ca\(^{2+}\)] studied. However, if M in the equations refers to more than one independent sites with affinity \(K_i/2\). Higher-order multimers would result in a greater separation of the observed affinities. We have therefore applied the minimal constraint \(K_s < K_i/4\).

The change in the amount of indicator bound to Ca\(^{2+}\) between diastole and systole for the five indicators (Table 1) is shown in Figure 3 (○) and the data for the [ATP] dependence of the ATPase obtained by Krause and Jacobus [15] are shown in Figure 4 (■). Constraining the model to fit the Ca\(^{2+}\) binding data from the NMR experiments alone does not enable us to calculate the affinities \(K_s\), \(K_s\), \(K_s\), and \(K_s\) unless we also specify the [ATP] dependence of Ca\(^{2+}\) binding to the myofibrils. This was achieved by simultaneously fitting the data in Table 1 and those from Krause and Jacobus by iteration of the variables in eqns. (8) and (4) respectively to minimize the combined SOS for both fits. This effectively constrains the apparent ATP dissociation constant of the myofilaments to 13.6 \(\mu\)M, when pCa = 4.5, as measured by Krause and Jacobus [15]. The line in Figure 3 shows the best fit of the Ca–ATP–Ca model to the NMR data and the line in Figure 4 the associated fit to the plot of the data for ATPase against [ATP].

The derivation of a continuous function for the best fit to the NMR data is given in the Computational methods section and in Figure 5.

The data fitting of the Ca–ATP–Ca model shown in Figures 3 and 4 yields values for the Ca\(^{2+}\)-affinities, \(K_s\), \(K_s\), and the product \(K_sK_s\) (see below). It is not possible to determine separate values for the affinities \(K_s\) and \(K_s\) because only data on the concentration of end product are available. In practice the constants \(K_s\) and \(K_s\) were allowed to vary independently in the model but we only report values for the product \(K_sK_s\). The fitting also gives values for the concentration of myofibrils [M] and the amount of Ca\(^{2+}\) released during the transient [Ca\(^{2+}\)], and sets limits on the parameters of other endogenous buffers. The model-derived parameters can be used to predict the effect of loading any concentration of any Ca\(^{2+}\) indicator on the cardiac Ca\(^{2+}\) transient.

In addition to the fitting of the empirical function to the diastolic NMR results, Figure 5 also shows the correlation of the derived systolic NMR data with those observed.

Values for the concentration and affinities of Ca\(^{2+}\) buffers in the intact heart derived from the new kinetic model of Ca\(^{2+}\) buffering

Concentration and affinity of myofibrillar Ca\(^{2+}\) binding sites

For the Ca–ATP–Ca model the data fitting fully utilized the constraint based on the interaction of two myofibril subunits, i.e. M has two troponin sites. The predicted affinities of the first and second ATP-independent Ca\(^{2+}\) bindings were \(10^4\) and
10^4 M^{-1} respectively. The apparent dissociation constant for the myofibrillar Ca^{2+} binding was 580 nM [in the presence of saturating phosphocreatine (PCr) and 5 mM ATP] and the concentration of Ca^{2+}-binding sites was 2 × 29 = 58 nM. The total mobilized Ca^{2+}, Δ[Ca]_m, was 44 μM. The value for logK_p/K_o, the ATP and subsequent Ca^{2+}-binding step, was 10.6. During low-stringency fitting the value of K_p rapidly approached 10^8 M^{-1} and increased to very high values as the stringency was increased.

In addition, if the two ATP-independent Ca^{2+}-binding steps (K_i and K_H) are replaced in the solution process with two non-interacting binding sites, i.e. using group constants without any constraint applied, these sites are found to be identical with a concentration of 29 μM and an affinity of 10^9 M^{-1}.

Comparison of the model predicted and measurements in vitro

Our model was constrained to fit data obtained under in vitro conditions, i.e. in the presence of PCr and [ATP] = 5 mM [22]. The data in vitro obtained by Holroyde et al. [11] for the Ca^{2+} dependence of the ATPase, however, were obtained in the absence of PCr and with [ATP] = 3 mM. Krause and Jacobus [15] showed that the [ATP] dependence of the ATPase is dependent on PCr. At pCa = 4.5 the [ATP] required for half activation of the myofibrils [Q = 0.5; eqn. (4)] was a minimal 13.6 μM in the presence of saturating PCr and 79.9 μM in the absence of PCr. The value obtained for K_p, K_o depends on this apparent ATP affinity [see eqn. (4)]. Therefore to reproduce the Ca^{2+} dependence of the ATPase under conditions in vitro we recalculated K_p/K_o for the absence of PCr. In addition to the absence of PCr the data of Holroyde et al. [11] were obtained at 4 mM [Mg^{2+}], whereas under the conditions of the NMR experiments (and therefore in our model) [Mg^{2+}], is 1.2 mM [23]. It has been shown that the apparent affinity of the ATPase for Ca^{2+} is [Mg^{2+}] dependent; a 5-fold increase in [Mg^{2+}] from 2 to 10 mM causes a 0.38 pCa shift in the apparent K_p for Ca^{2+} of the ATPase [24]. Therefore, assuming that the apparent Ca^{2+} dissociation constant has linear dependence on [Mg^{2+}] over the range 1–10 mM, we have generated values for the data of Holroyde et al. [11] corrected to that expected for a [Mg^{2+}] of 1.2 mM (see the text for details).

Additional endogenous buffers

For the Ca–ATP–Ca model the predicted affinity and/or concentration of the additional endogenous buffer, K_p and [E]_m, are too low to make any significant contribution to Ca^{2+} buffering. These results rule out other endogenous non-co-operative cytosolic Ca^{2+} buffers from contributing to buffering of the cardiac Ca^{2+} transient. However, if the additional buffer were cooperative with two sites for Ca^{2+} with an apparent dissociation constant close to that of the myofibrils, this buffer could be included in the total binding sites of 58 μM, with the myofibrils making up one component and the additional buffer the remainder. It is not possible within the limits of the experimental errors of our NMR measurements to determine whether the buffering is composed of a single component or two components with similar characteristics. Solutions to the data fitting when an additional buffer with co-operative binding is included do show minimization of the SOS as its concentration approaches zero, albeit with a very small gradient of approach to minimum (Figure 7).

Effect of the indicator concentration on the Ca^{2+} transient

To obtain adequate S/N ratios, 19F-NMR indicators have to be loaded to concentrations of approx. 120 μM, which clearly perturbs the [Ca^{2+}] transient [2]. We can use the model to calculate the perturbation of the Ca^{2+} transient that would occur for any Ca^{2+} indicator at any concentration. Figure 8 shows the predicted effect of loading an indicator with a K_d of 220 nM at intracellular concentrations of 0–250 μM. The model predicts that even at 30 μM an indicator with K_d = 220 nM would reduce...
of the acetoxymethyl esters in the cytosol [25–27].

indicators into the heart, reflecting a constant rate of hydrolysis reflects the time course of developed pressure found when loading reproduce our of the data with a single site for Ca

Previous estimates of Ca

New kinetic model of Ca

DISCUSSION

New kinetic model of Ca

Figure 7 Additional endogenous buffers

The solution process for the Ca–ATP–Ca model resulted in a fit of the data that was just within the random experimental error. This allows for little or no additional endogenous buffering. If we assume a cumulative error resulting from approximations made, then a limit to the other endogenous buffers can be estimated as follows: the solution results are taken as correct within the context of the accumulated error; the additional buffering that can be added keeping the solution to these values within the random experimental error may then be estimated. Shown are the maximum extra 1:1 (dotted line) or 2:1 (broken line) endogenous Ca

Figure 8 Effect of Ca

The expected [Ca

the [Ca

50%. It is noted that Figure 8 reflects the time course of developed pressure found when loading indicators into the heart, reflecting a constant rate of hydrolysis of the acetoxymethyl esters in the cytosol [25–27].

DISCUSSION

New kinetic model of Ca

Previous estimates of Ca

the intact heart. In formulating the model we have also taken into account the constraints imposed by the kinetics of cardiac contraction, which dictate that the rates of dissociation of Ca

from the myofibrils must be at least 50 s

and that Ca

binding to the myofibrils must be ATP dependent. These considerations have led us to propose the model, depicted in Scheme 2, in which there is ATP-dependent Ca

binding to the myofibrils, the principal cytosolic Ca

buffer. In the model, a first Ca

is bound, followed by ATP, which effectively raises the affinity of a second Ca

binding from low to high. The second Ca

is then bound. Hydrolysis of the ATP (with a rate constant of 65 s

[18]) causes the second Ca

binding site to revert to its low-affinity state, thereby enabling the myofibrils to relax through rapid release of Ca

Estimation of Ca

buffering in the intact heart

The new kinetic model of Ca

buffering was constrained to fit our data for the

F-NMR measurement of [Ca

], in the Langendorff-perfused ferret heart as well as the data in vitro for the [ATP] dependence of the myofibrillar ATPase [15] (see Figures 3 and 4). Under these constraints, a value was derived for the concentration of the myofibrillar Ca

-binding sites of 58 M

with half activation of the ATPase at 580 nM. Solaro et al. [29] estimated the concentration of high-affinity Ca

-specific binding sites in ventricular muscle to be approx. 70 M, which is close to the value derived from our model.

To estimate the concentration and affinities of endogenous Ca

buffers it was necessary to know how much Ca

was released into the cytoplasm during each Ca

transient. To allow for the possibility that the depolarization-induced Ca

release may vary with the increased buffering imposed by loading a Ca

indicator, a ‘Ca

store’ with internal concentration [Ca

], and a release dependent on the difference, [Ca

] – [Ca

], was included in the model as a mathematical device. For such a store, if [Ca

] is close to [Ca

], then the driving force for Ca

release will be small and the amount released will be highly dependent on [Ca

], and therefore on the presence of Ca

indicators if they perturb the [Ca

]. However, if [Ca

] is high relative to [Ca

], then the driving force for release will be large and the amount of Ca

released will be effectively independent of [Ca

]. In solutions to our model the amount of Ca

mobilized remained constant, 44 M for the Ca–ATP–Ca model, which suggests that there is no increase in mobilized Ca

induced by the indicator. This prediction is consistent with the recent estimation of the concentration of free Ca

in the sarcoplasmic reticulum of approx. 700 nM buffered by 14 mM binding sites with K

= 640 M [30]. It is also in agreement with the study from Adachi-Akahane et al. [31], who showed that the amount of Ca

released from the sarcoplasmic reticulum was unaffected by increasing the cytosolic Ca

buffering to 16 mM (2 mM fura-2 plus 14 mM EGTA). Estimates from the model of 40–44 M mobilized Ca

are very similar to the recent estimates of Ca

release in ferret ventricular myocytes of 30–60 M [32] but are less than those estimated in many previous studies (typically 100–130 M [7,10,33]). It is possible that the earlier studies overestimated the release of Ca

because they assumed much higher values for Ca

buffering than were calculated in our model. It is also possible that species variations, which are known to contribute to differences in Ca

handling in the heart, may provide part of the explanation [34].

Endogenous buffers

The Ca–ATP–Ca model was able to fit our experimental data to within experimental error without the need to include any
buffering other than the myofibrils. However, this does not exclude the possibility that other buffers present could contribute to the total. The $K_v$ values for the four calmodulin Ca$^{2+}$-binding sites, when measured under in vivo conditions, all have values well below 100 nM and slow dissociation rates [5]; they will therefore not contribute significantly to Ca$^{2+}$ buffering. This is consistent with calmodulin having a control function that depends on the ‘average’ and not the cycling [Ca$^{2+}$]. The $K_v$ for the sarcolemmal Ca$^{2+}$-binding sites has been estimated to be 180 nM [9]. However, the voltage dependence of this Ca$^{2+}$-binding site (i.e. depolarization causes a reduction in affinity, which would result in release of Ca$^{2+}$ rather than binding of Ca$^{2+}$ during the transient [35]) makes this site unlikely to contribute to Ca$^{2+}$ buffering. It has recently been shown that Ca$^{2+}$ reuptake by the sarcoplasmic reticulum could be best described by a process that binds Ca$^{2+}$ with a Hill coefficient of 2 and is half-saturated when [Ca$^{2+}$] = 500 nM [36]. These parameters would enable the sarcoplasmic reticulum to make a significant contribution to Ca$^{2+}$ buffering during the cardiac Ca$^{2+}$ transient. However, the sarcoplasmic reticulum also releases Ca$^{2+}$ during the ascending phase of the transient and its Ca$^{2+}$ binding may follow that of the sarcolemma. The estimated concentration of sarcoplasmic reticulum Ca$^{2+}$ uptake sites of 19 $\mu$M [10] could be included within that estimated here.

In the present study the approximation that all indicators were loaded to the same concentration of 120 $\mu$M is a potential source of inaccuracy in the calculation of the endogenous buffering present in the heart. However, as 120 $\mu$M is two to three times the estimated concentration of endogenous buffers any errors would occur in the estimation of the total concentration of Ca$^{2+}$-binding sites but have little effect on the estimated affinity of those sites.

Effect of loading Ca$^{2+}$ indicators on cardiac Ca$^{2+}$ transients

A useful prediction of the model is the extent of perturbation of the Ca$^{2+}$ transient expected on introducing additional exogenous buffers, such as Ca$^{2+}$ indicators. For the simplest model, i.e. containing only the myofibrils, as little as 30 $\mu$M of an indicator with a $K_v$ of 220 nM would cause more than 50% reduction in the magnitude of the Ca$^{2+}$ transient (see Figure 8). In most studies where [Ca$^{2+}$] is measured using spectroscopic Ca$^{2+}$ indicators there has been no estimation of the concentration of indicator present in the cytosol, although it appears that the concentrations used range from 30 to 900 $\mu$M [4]. As well as causing significant perturbations of the [Ca$^{2+}$], such concentrations of added indicators will also slow the transients (e.g. [27]) and therefore complicate kinetic analysis of Ca$^{2+}$ handling in the heart. Significant perturbation of Ca$^{2+}$ transients caused by loading Ca$^{2+}$ indicators is not limited to cardiac muscle (see Zhou and Neher [37], who noted similar perturbations in chromaffin cells).

Hill coefficients of the myofibrillar ATPase and 45Ca binding

A conventional model of cardiac myofibrillar ATPase activity, i.e. one that included a single Ca$^{2+}$-binding site and an ATP-binding step, was unable to reproduce our experimental data (see Figure 3). Conversely, a model that included ATP-dependent cooperative Ca$^{2+}$ binding to the myofilaments was able to reproduce our experimental data. Furthermore, this model was able to predict values for the Ca$^{2+}$ dependency of the myofibrillar ATPase that closely agreed with the experimental data of Holroyde et al. [11] (see Figure 6).

The model presented here explains the increased co-operativity of 45Ca binding in the presence of ATP, although this is not accompanied by a clear increase in numbers of Ca$^{2+}$ bound. In the absence of ATP the species formed is MCa$_2$, where M is a dimeric unit and the affinities show no co-operativity. In the presence of ATP the species formed (MCa$_2$ATP) has high Ca$^{2+}$ co-operativity but again only one Ca$^{2+}$ per subunit. The results in this paper introduce an unexpected mode of interaction between subunits of the myofilibr to answer the question of how the apparent co-operativity of Ca$^{2+}$ activation could arise. It could arise either at the Ca$^{2+}$-binding level or, as previously argued, after substrate binding. If it arose after substrate binding, e.g. via the interaction of two activated actomyosin subunits, then there would be no need to invoke a second Ca$^{2+}$-binding site per ATPase. It would then be expected that, in addition to the apparent Hill coefficient for the [Ca$^{2+}$] dependency of the ATPase being greater than 1 (see Figure 2), the Hill coefficient for the [ATP] dependence of the ATPase activity would also be greater than 1. The Hill coefficient for [ATP], however, is unity (see Figure 4), so we can exclude the possibility that any of the apparent higher order for Ca$^{2+}$ dependency is due to positive co-operativity between ATPases or with cross bridges. The present model requires that cardiac myofibrils have a second Ca$^{2+}$-binding site on an interacting subunit with an apparent affinity that is modulated by ATP binding. Expressed simply, the binding of ATP reduces the affinity of interacting subunits to bind the first Ca$^{2+}$ and increases that for the second. Importantly, only one ATP is hydrolysed by the two interacting subunits.

The situation is similar in the co-operative Ca$^{2+}$ binding by, and Ca$^{2+}$ activation of, myofibrils from skeletal muscle [38]. These have one major difference from the cardiac myofibrils: skeletal muscle troponin C is known to have two Ca$^{2+}$-specific binding sites with dissociation rates in the physiologically relevant range, whereas cardiac troponin C has only one [9,11,12].

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