Ca²⁺ buffering in the heart: Ca²⁺ binding to and activation of cardiac myofibrils

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The measurement of cardiac Ca²⁺ transients using spectroscopic Ca²⁺ indicators is significantly affected by the buffering properties of the indicators. The aim of the present study was to construct a model of cardiac Ca²⁺ buffering that satisfied the kinetic constraints imposed by the maximum attainable rates of cardiac contraction and relaxation on the Ca²⁺ dissociation rate constants and which would account for the observed effects of ¹⁹F-NMR indicators on the cardiac Ca²⁺ transient in the Langendorff-perfused ferret heart. It is generally assumed that the Ca²⁺ dependency of myofibril activation in cardiac myocytes is mediated by a single Ca²⁺ binding site on troponin-C. A model based on 1:1 Ca²⁺ binding to the myofilaments, however, was unable to reproduce our experimental data, but a model in which we assumed ATP-dependent cooperative Ca²⁺ 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 Ca²⁺ binding, giving 58μM

and 2.0μM respectively. In addition to reproducing our experimental data on the concentration of free Ca²⁺ ions in the cytoplasm ($[Ca^{2+}]_i$), the resulting Ca²⁺ and ATP affinities given by fitting of the model also provided good predictions of the Ca²⁺ dependence of the myofibrillar ATPase activity measured under *in vitro* conditions. Solutions to the model also indicate that the Ca²⁺ mobilized during each beat remains unchanged in the presence of the additional buffering load from Ca²⁺ indicators. The new model was used to estimate the extent of perturbation of the Ca²⁺ transient caused by different concentrations of indicators. As little as 10μM of a Ca²⁺ indicator with a dissociation constant of 200nM will cause 20% reduction in peak-systolic $[Ca^{2+}]_i$ and 30μM will cause $\approx$50% reduction in the peak-systolic $[Ca^{2+}]_i$ in a heart paced at 1.0Hz.

Key words:
Ca²⁺-transient, Troponin, Actomyosin-ATPase, Kinetics, Calcium metabolism

Introduction

The concentration of free calcium 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. 160nM to a systolic value of approx. 2700nM [2]. These values are determined by the amount of Ca²⁺ entering and leaving the cytosol during each cardiac cycle and the kinetic properties of the cytoplasmic Ca²⁺ buffers. Most information on cardiac $[Ca^{2+}]_i$ has come from studies using spectroscopic Ca²⁺ indicators (e.g. [2,3] and references therein).

These indicators can accurately report $[Ca^{2+}]_i$ in biological tissues, but they also chelate Ca²⁺ 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 Ca²⁺ buffers in the heart may be divided into three groups based on their affinities. High affinity buffers (association constant (K_a)>3x10⁷M⁻¹, e.g. calmodulin; [5]) will be >80% bound in diastole

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Abbreviations used:

ATP refers generally to the total adenosine triphosphate in the presence of at least 1mM Mg²⁺, i.e. near saturated [MgATP²⁻];

$[Ca^{2+}]_i$, concentration of free Ca²⁺ ions in the cytoplasm;

PCr, phosphocreatine;   SOS, sum of squares;

DiMe-5FBAPTA, 1,2-*bis*-[2-(1-carboxyethyl)(carboxymethyl)amino-5-fluorophenoxy]ethane;

DiMe-4FBAPTA, 1,2-*bis*-[2-(1-carboxyethyl)(carboxymethyl)amino-4-fluorophenoxy]-ethane;

5FBAPTA, 1,2-*bis*-[2-*bis*(carboxymethyl)amino-5-fluorophenoxy]ethane;

4,5FBAPTA, 1,2-*bis*-[2-*bis*(carboxymethyl)amino-4,5-difluorophenoxy]ethane;

MFBAPTA, 1,2-*bis*-[2-*bis*(carboxymethyl)amino-5fluoro-4-methylphenoxy]ethane.

K_a association constant (affinity)

K_d dissociation constant

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and will not contribute significantly to buffering of the cardiac Ca^{2+} transient. Conversely, buffers with low affinities (association constant $K_a < 10^4 M^{-1}$) 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–$10^7 M^{-1}$. 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 250–300μM [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 123μM with an apparent dissociation constant of 0.96μM [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+} cooperatively, but still only one per troponin-C, with an apparent K_d in the μM 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 cooperative 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 data base

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 concentration of ^{3}H-labelled 1,2-*bis*-[2-*bis*(carboxymethyl)amino-5-fluorophenoxy]ethane (5FBAPTA) in hearts with signal-to-noise (S:N) ratio of 10:1 was approximately 120μM [14]. All indicators were loaded until the S:N ratio was approximately 10:1; we therefore have assumed that all indicators were present at a concentration of 120μM [2]. The differences in the buffering of the Ca^{2+}-transient between the different indicators will therefore predominantly reflect differences in their Ca^{2+}-affinities. The experimental determinations of diastolic and systolic $[Ca^{2+}]_i$ in the perfused ferret heart, for indicators with K_d values ranging from 46nM to 2950nM are summarized in Table 1.

The data sets used in this study were acquired under two limiting sets of conditions. The $[Ca^{2+}]$ 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 $[Ca^{2+}]_i$ in the perfused heart were made under near steady-state conditions (see [2]), i.e. the rate of data block collection ($16s^{-1}$) was much slower than the rate limiting step of the isometrically limited ATPase ($65s^{-1}$, see below). The rate of data collection was also rapid compared to the rise and fall of $[Ca^{2+}]_i$ [2].

Indicator	K_d at 30°C (nM)	Titration range (nM)	Indicator bound (%)	
			Diastole	Systole
4,5FBAPTA	2950	520–17,000	17	46
5FBAPTA	537	100–3,000	49	77
MFBAPTA	269	40–1,100	56	*
DiMe-4FBAPTA	155	30–900	65	82
DiMe-5FBAPTA	46	8–300	79	*

Table 1: Summary of data for diastolic and systolic $[Ca^{2+}]_i$ in Langendorff-perfused ferret hearts loaded with different ^{19}F-NMR Ca^{2+} indicators[2].
* These values were not determined experimentally. They were therefore allowed to vary to make minimal direct contribution to the fitting process. However, the values obtained were included in extrapolations to obtain the unperturbed diastolic and systolic $[Ca^{2+}]_i$ that were used to calculate the mobilized Ca^{2+} in the fitting process (see Figure 3 for details).

We have therefore used equilibrium kinetics to derive the model for Ca^{2+} buffering in the heart.

In the isotonic case there is also no external work performed; 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 has allowed the use of the same equilibrium equations to generate the model predicted $[Ca^{2+}]$ and $[ATP]$ dependencies of the ATPase (see the Results section).

Construction of the new kinetic model of Ca^{2+}-buffering in the intact heart is based in part qualitatively on data for the $[Ca^{2+}]$ 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 used with permission of the authors).

Computational methods

Solutions to the models were found by an iterative alteration of the unknown variables until the sums of squares (SOS) 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, USA).

The only constraints imposed were that all parameters be positive. The functions to be minimized were highly nonlinear 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 (p. 574, Microsoft Excel Users Guide). 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 Ca^{2+} in diastole ($[FCa]_D/[F]_T$) with indicator affinity ($\log K$) was found by fitting those data (Table 1) to an arbitrary two term sigmoidal function, $[FCa]_D/[F]_T = 1/(1+a^{(b-\log K)}+c^{(d-\log K)})$, shown as solid line 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 $\log K$ (see Figure 3) and the expected fraction bound (dotted line 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 Ca^{2+} activation of the myofibrils in vitro *is greater than one*

The data for $[Ca^{2+}]$-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 cooperative 2:1 binding of Ca^{2+} to the myofibrils (dotted line and solid line respectively) based on the binding Schemes shown in Scheme 1 (below) for regulation by Ca^{2+}, in which M represents the myofibrils and K_1 and K_2 are the affinity constants for the first and second ions bound.
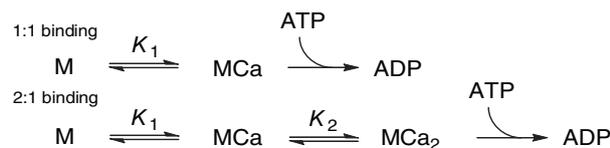
Scheme 1:

The 1:1 binding provides a very poor fit to the data, this indicates that the process exhibits cooperativity. The 2:1 model provides a much better fit, although at high $[Ca^{2+}]$ where the data will be most accurate,

a model with more than two Ca^{2+} binding would fit better. Therefore, the myofibrillar ATPase data of Holroyde *et al.*[11] indicate that Ca^{2+} binding to the myofibrils *in vitro* is strongly cooperative so the concentration of singly ligated myofibrillar protein will be small.

Limitations of the 2:1 Ca^{2+} binding model: the affinities of individual Ca^{2+} 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: *(Equation 1)*

$$v = V_{max} \frac{K_1 K_2 [Ca^{2+}]^2}{1 + K_1 [Ca^{2+}] + K_1 K_2 [Ca^{2+}]^2}$$
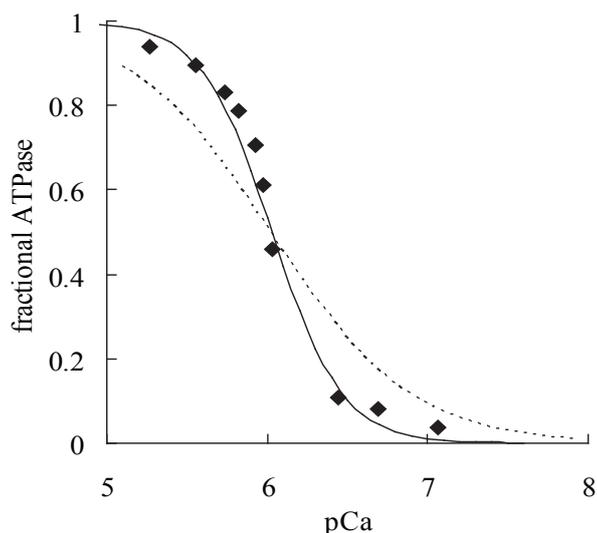
Figure 1: Ca²⁺ dependence of myofibrillar ATPase
Data points (◆) are reproduced, with permission, from Holroyde *et al.* [11]. Lines represent best fits to the data for 1:1 Ca²⁺ (dotted line) and 2:1 Ca²⁺ (solid line) binding Schemes as described in Scheme (1) (see the text). The [Ca²⁺] at which the activity of the ATPase is half maximal is $10^{-6.1}$M.

Figure 2: Limits to the affinities for individual binding steps for the 2:1 Ca²⁺ binding Scheme
The effect of fixing the value for the first Ca²⁺ affinity, K_1, in the range 10^3–10^9M⁻¹ for equation (2), in which the ATPase is proportional to [MCa₂]. Right ordinate (broken line), the value found for K_2 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 ensure that the first Ca²⁺ could not dissociate, which would make the process effectively first order, already shown to give a very poor fit to the data (Figure 1).

where K_1 and K_2 represent the affinities of the two Ca²⁺ 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 (K_2) step 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 (10^3–10^9M⁻¹) and the corresponding deviations (SOSs) from ideal fit are shown in Figure 2. At values of $K_1 > 10^5$M⁻¹ the SOS increased significantly. This suggests that if Ca²⁺ binding to the myofibrils follows the simple two step Ca²⁺ binding Scheme, as described by Equation 1, then for this model K_1 must be $<10^5$M⁻¹ and therefore K_2 must be more than 10^7M⁻¹ (see Figure 2). This is not consistent with the Ca²⁺ affinity of troponin-C ($10^{5.6}$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 3–10Hz (depending on species). For a rate of 5Hz (the maximum observed for ferret; [16]) this corresponds to systolic and diastolic durations of less than 100ms each. To allow full relaxation of the myofibrils more than 95% of the second Ca²⁺ must have dissociated from the myofibrils, i.e. the dissociation rate must be more than 50s⁻¹. If we assume that the Ca²⁺ on-rate for the myofibrils is maximal, i.e. limited by diffusion at $\approx 5 \times 10^8$M⁻¹·s⁻¹ [17], then the upper limit

for the affinity of the second Ca²⁺ binding step (K_2) would be $5 \times 10^8/50$, i.e. 10^7M⁻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 10^7M⁻¹, is not compatible with the kinetic constraints of the rates of contraction and relaxation of the myofibrils. This means that Ca²⁺ binding to the myofibrils cannot be explained by the simple two-step binding process described in Equation 1. By analogous reasoning Ca²⁺ binding to the myofibrils cannot be explained by a third or higher order allosteric regulated binding Scheme. Therefore, given that Ca²⁺ binding to the myofibrils is cooperative (see Figure 1), Ca²⁺ binding to the myofibrils must be modulated by other factors.

Limitations of the 2:1 Ca²⁺ binding model: the Hill coefficient for ATP activation of the myofibrillar ATPase is unity

It has previously been proposed that cooperativity between cross-bridges could explain the high Hill coefficient of the [Ca²⁺] activation of the ATPase. This would be equivalent to M (in Scheme 1) being two separate enzyme units both binding Ca²⁺ and ATP and working in concert. This mechanism is inconsistent with the lack of cooperativity seen for the [ATP] dependency of the ATPase (see the Discussion section). In addition, the limits to Ca²⁺ affinity set by the maximum cardiac rate would also apply for this model.

Resolution of the simple model problems

Modulation of Ca^{2+} binding by ATP

The obvious candidate for modulation of 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 myofibrillar ATPase was the rate of ADP dissociation which has a rate constant of $65s^{-1}$. The similarity of the rate of ATP hydrolysis with the minimum constraint imposed by the rate of relaxation ($50s^{-1}$, 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. 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. $65s^{-1}$. In effect, the affinity of the Ca^{2+} binding could be much greater than 10^7M^{-1} in the presence of bound ATP, yet still have an effective dissociation rate of $\approx 50s^{-1}$.

Recent ^{45}Ca binding studies have shown that in the presence of ATP, Ca^{2+} binding to the myofibrils has increased cooperativity [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 cooperativity 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 cooperative $[Ca^{2+}]$ 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_p and K_3 in Scheme 2. This we shall refer to as the Ca–ATP–Ca model.
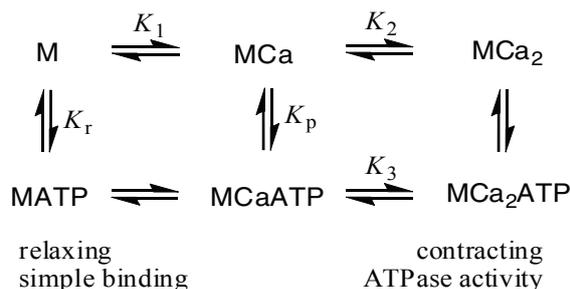
(Scheme 2:)

Scheme 2: Ca–ATP–Ca model of Ca^{2+} and ATP binding to the myofibrils

The simplest model based on the ATP-dependent cooperative Ca^{2+}-binding observed by Morimoto and Ohtsuki [13] is represented by the following steps; K_1, The Ca^{2+}-free myofilament (M) binds the first Ca^{2+} ion; K_p, the myofilament with one bound Ca^{2+} binds ATP; K_3, a second Ca^{2+} is bound. The cooperativity of Ca^{2+} binding requires $K_3 > K_1 > (K_2)/4$ resulting in a strongly bound ATP state generating the ATPase activity The full Scheme incorporates the pre ATP binding of the second calcium (K_2) which is required to give the limiting value for ATP apparent affinity at high $[Ca^{2+}]$. Effectively the affinity of the second Ca^{2+} binding (K_2) is increased (to K_3) by the prior binding of ATP. Also included is the Ca^{2+} independent ATP binding (K_r) which has been equated to the "loosely bound ATP state" and results in relaxation of the protein.

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

In addition to the basic Scheme (K_1–K_p–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:

1. 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 cooperativity [19] implies ATP binding before the second Ca^{2+}. The Scheme allows a second Ca^{2+} to bind before ATP (K_2, in Scheme 2), as well as after ATP (K_3). For this Scheme the apparent ATP affinity at very high $[Ca^{2+}]$ will be $K_p \cdot K_3/K_2$. Without the ATP-independent Ca^{2+} binding, i.e. if K_2 were to be zero, the apparent affinity for ATP would increase indefinitely with $[Ca^{2+}]$.

2. The myofibrils can bind ATP in the absence of Ca^{2+} (K_r, in Figure 3), termed the loose-bound ATP state [21]. The value of K_r, measured from the ATP-dependent changes in fluorescence of the isolated myofibrils is $\approx 10^3M^{-1}$ [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 cooperative 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 Ca²⁺ buffering in the intact heart

Although the myofibrils represent the major source of Ca²⁺ binding during the cardiac Ca²⁺ transient, any model of Ca²⁺ buffering in the intact heart must also consider the contributions of other endogenous buffers and any exogenous buffers (e.g. Ca²⁺ indicators loaded into the heart). Furthermore to calculate the buffering of Ca²⁺ in the intact heart it is also necessary to know how much Ca²⁺ enters the cytoplasm (from extracellular as well as intracellular stores) during each contraction.

Ca²⁺ binding to the myofibrils

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

$$[M]_T = [M]\left(\begin{array}{l} 1 + K_1[Ca^{2+}] + K_r[ATP] + K_1 K_P[ATP][Ca^{2+}] \\ + K_1 K_2[Ca^{2+}]^2 + K_1 K_3 K_P[ATP][Ca^{2+}]^2 \end{array}\right)$$

where K_1, K_2, K_3 and K_r, K_p are the affinity constants for the myofibrillar Ca²⁺ 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 Ca²⁺-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;   *(Equation 3)*

$$[M]_T = M\left(\alpha + \beta[Ca^{2+}] + \gamma[Ca^{2+}]^2\right) \quad \text{where} \quad \begin{array}{l} \alpha = 1 + K_r[ATP] \\ \beta = K_1 + K_1 K_P[ATP] \\ \gamma = K_1\left(K_2 + K_3 K_P[ATP]\right) \end{array}$$

and hence we can obtain an expression for the fraction of the myofibrils activated as MCa₂ATP, denoted Q, which for the case of the Ca–ATP–Ca model (Scheme 2) is: *(Equation 4)*

$$Q = \frac{K_1 K_3 K_P[ATP][Ca^{2+}]^2}{\alpha + \beta[Ca^{2+}] + \gamma[Ca^{2+}]^2}$$

Ca²⁺ binding to other endogenous Ca²⁺ buffers

Other non-myofibrillar Ca²⁺ binding species, e.g. calmodulin and proteins on the cytoplasmic leaflets of both the sarcolemma and the sarcoplasmic reticulum membranes [9,10], may also contribute to Ca²⁺-buffering in the intact heart. If we assume that the additional endogenous Ca²⁺ buffers can be considered as a single species that binds one Ca²⁺ ion, then the following expression for the total calcium, $[Ca]_T$, in the cytoplasm can be derived:

(Equation 5)

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

where $[E]_T$ is the total amount of other endogenous buffers attributed an affinity constant of K_E.

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 Ca²⁺ buffers bind Ca²⁺ cooperatively (see the Discussion section).

Ca²⁺ binding to exogenous Ca²⁺ buffers (Ca²⁺ indicators)

To calculate Ca²⁺ buffering in the presence of Ca²⁺ indicators we measure the change in the fraction of indicator bound to Ca²⁺ between diastole and systole [2], $\Delta[FCa]/[F]_T$, i.e.:   *(Equation 6)*

$$\Delta[FCa]/[F]_T = \left(\frac{[FCa]_S}{[F]_T} - \frac{[FCa]_D}{[F]_T}\right)$$

where $[FCa]$ and $[F]_T$ are the concentrations of Ca²⁺ bound and total indicator. The subscripts S and D refer to systole and diastole.

Ca²⁺ entry into the cytoplasm during the Ca²⁺ transient

To calculate the buffering of $[Ca^{2+}]_i$ in the intact heart it is necessary to know how much Ca²⁺ enters the cytoplasm ($\Delta[Ca]_T$) as well as the change in $[Ca^{2+}]_i$ during each contraction. For the purposes of the model it is assumed that all sources of Ca²⁺ entry (from extracellular as well as intracellular sources) can be combined into a single Ca²⁺ source. As a first approximation we have assumed that the total Ca²⁺ mobilized during the cardiac cycle is independent of total (endogenous + exogenous) cytosolic Ca²⁺ buffering (see Discussion). $\Delta[Ca]_T$ can then be calculated as the sum of the changes in $[Ca^{2+}]_i$, Ca²⁺ bound to the myofibrils, Ca²⁺ bound to other endogenous buffers, and Ca²⁺ bound to the indicator, between diastole and systole: *(Equation 7)*

$$\Delta[Ca]_T = \left\{\begin{array}{l} [Ca^{2+}]_S - [Ca^{2+}]_D + \left(\sum[MCa]_S - \sum[MCa]_D\right) \\ + 2\left(\sum[MCa_2]_S - \sum[MCa_2]_D\right) + \left([ECa]_S - [ECa]_D\right) + \Delta[FCa] \end{array}\right\}$$

The $\sum$ terms are used to indicate all species that are bound to either one or two Ca²⁺ 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: $[Ca^{2+}]$, $[ATP]$ and K_r, and the seven unknowns, *viz.* $\Delta[Ca]_T$, $[M]_T$, $[E]_T$, K_1, K_2, K_3 and K_p

which can be expressed as a function of the measured variable, the change in fraction of indicator bound to Ca^{2+}, $\Delta[FCa]/[F]_T$: *(see Equation 8)* This equation therefore represents the new kinetic model of Ca^{2+} buffering in the intact heart that we have used to fit the NMR data for indicator K_d-dependent decline in $[Ca^{2+}]_i$ and thereby to estimate parameters for Ca^{2+} binding to the myofibrils and other endogenous Ca^{2+} buffers in the heart.

(Equation 8)

$$\Delta\frac{[FCa]}{[F]_T} = \left(\begin{array}{c} \dfrac{\Delta[Ca]_T}{[F]_T} - \dfrac{[Ca^{2+}]_S}{[F]_T}\left\{1 + \dfrac{[M]_T\left(\beta + 2\gamma[Ca^{2+}]_S\right)}{\left(\alpha + \beta[Ca^{2+}]_S + \gamma[Ca^{2+}]_S^2\right)} + \dfrac{[E]_T K_E[Ca^{2+}]_S}{\left(1 + K_E[Ca^{2+}]_S\right)}\right\} \\[4mm] + \dfrac{[Ca^{2+}]_D}{[F]_T}\left\{1 + \dfrac{[M]_T\left(\beta + 2\gamma[Ca^{2+}]_D\right)}{\left(\alpha + \beta[Ca^{2+}]_D + \gamma[Ca^2]_D^2\right)} + \dfrac{[E]_T K_E[Ca^{2+}]_D}{\left(1 + K_E[Ca^{2+}]_D\right)}\right\} \end{array}\right)$$

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

To determine the parameters for Ca^{2+} buffering in the intact heart we constrained the model to fit two independent sets of results: first, the changes in the Ca^{2+}-transient following the loading of ^{19}F-NMR Ca^{2+} indicators in the Langendorff-perfused ferret heart [2] and second, the [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 $[P_i]$ 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 Ca^{2+} bound, to the active pool. The prerequisite for the maximum possible rate of relaxation of the myofibrils is that the dissociation rates of the Ca^{2+} binding steps (K_1 and K_2), and consequently any subsequent ATP binding, must be faster than $50s^{-1}$. 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$_2$ATP species and the loss of this species by the rate limiting ADP dissociation. However, in the steady state the concentration of MCa$_2$ATP will be proportional to its rate of formation, i.e. the concentration of the precursor (MCaATP) multiplied by the $[Ca^{2+}]$ and a constant. If normalized rates (fraction of maximal ATPase) are used then the constant disappears from the numerator of Equation 4. The rate limiting step returns the myofibril to the pool equilibrating with Ca^{2+} and ATP

bound species and is included as MCa$_2$ATP in the sum of species making up the total myofibril concentration in the denominator of Equation 4. Therefore, with the above considerations we may use Equation 4 for 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 [MCa$_2$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 cooperative [13] which would seem to be at odds with the model derived above, unless $K_2 \ll K_1$ and not titrated in the range of $[Ca^{2+}]$ studied. However, if M in the equations refers to more than one unit then for the two-sub unit case where there is no positive cooperativity, K_2 cannot exceed $K_1/4$ (the result of two independent sites with affinity $K_1/2$). Higher order multimers would result in a greater separation of the observed affinities. We have therefore applied the minimal constraint $K_2 < K_1/4$.

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_1, K_2, K_3 and K_p 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 Equations 8 and 4, respectively, to minimize the combined SOS for both fits.

This effectively constrains the apparent ATP dissociation constant of the myofilaments to 13.6μM, 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 ATPase against [ATP].
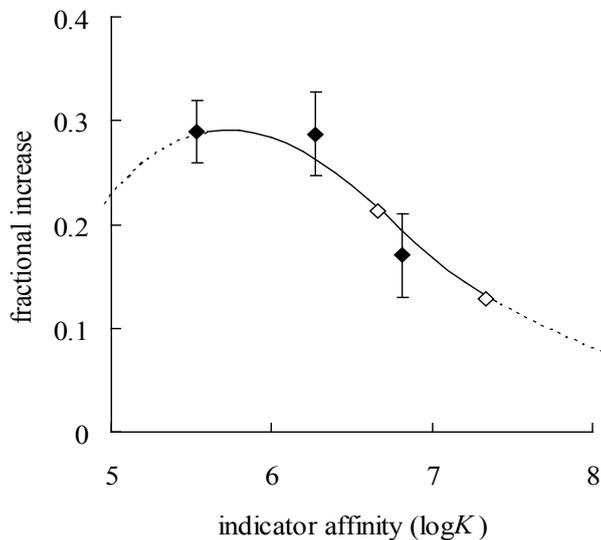
Figure 3: The increase between diastole and systole in the fraction of ^{19}F indicator that is Ca^{2+} bound

The changes observed are shown (■). The line represents best fit for the Ca–ATP–Ca model. Values for the systolic [Ca^{2+}]$_i$ in the presence of 1,2-*bis*-[2-*bis*(carboxymethyl) amino-5-fluoro-4-methylhenoxy]ethane (MFBAPTA) and 1,2-*bis*-[2-(1-carboxyethyl)(carboxymethyl)amino-4-fluoro-phenoxy]ethane (DiMe-4FBAPTA) (◇) 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+}]$_i$ for all indicators, however, were included in the extrapolation process to determine the unperturbed systolic and diastolic [Ca^{2+}]$_i$ in each model solution. In all cases the values for unperturbed diastolic and systolic [Ca^{2+}]$_i$ for the best fits of the models were in the range 150±10nM and 2670±50nM respectively. Both values are very similar to those we 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, therefore, the resultant curve is plotted as dotted line in these regions. However, as expected the increase tends to zero each side of the data range available.
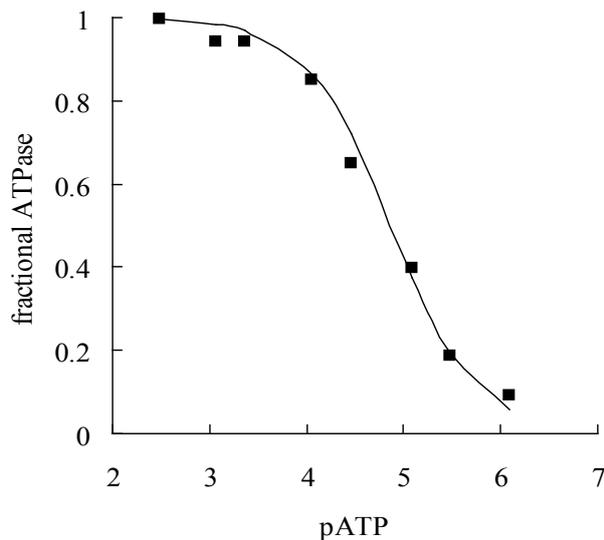
Figure 4: Constraining the models to the [ATP] against ATPase data

Data for the [ATP] dependence of the myofibrillar ATPase in the presence of PCr (■) (re-plotted from Krause and Jacobus [15] with the permission of the authors). The line of best fit was calculated from the Ca–ATP–Ca model. The SOS of the errors between the model calculated values (solid line) and the experimental values (■) for the ATPase were combined with the SOS derived from the NMR data (shown in Figure 3). Scaling was applied so that each set of SOS would make approximately equal contribution to the final SOS. The best fits shown in both Figure 3 and 4 were thence obtained by minimising the combined SOS.

The derivation of a continuous function for the best fit to the NMR data is given in the Computational Methods section and 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_1, K_2 and the product K_3K_p (see below). It is not possible to determine separate values for the affinities K_3 and K_p as only data on the concentration of end product are available. In practice the constants K_3 and K_p were allowed to vary independently in the model but we only report values for the product K_3K_p. The fitting also gives values for the concentration of myofibrils [M]$_T$, the amount of calcium released during the transient Δ[Ca]$_T$ 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.
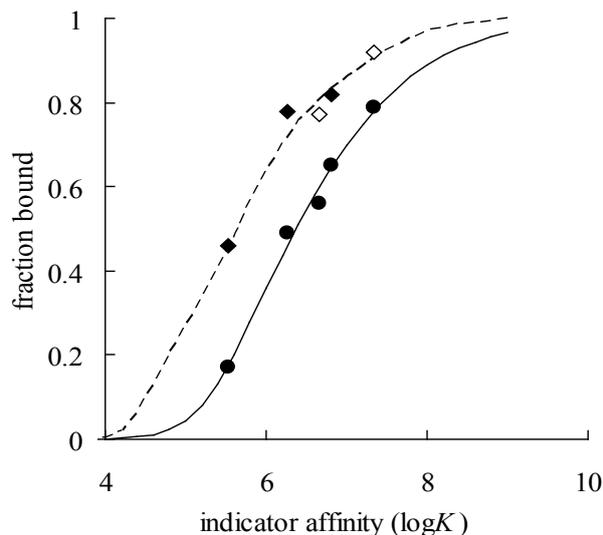
Figure 5:
Derivation of continuous functions for fraction of indicator bound to Ca^{2+} plotted against indicator K_d
The data from Table 1 are shown [diastolic (●) and systolic (◆)]. The solid line is the best fit of the arbitrary two term sigmoidal function, $[FCa]_D/[F]_T = 1/(1+a^{(b-logK)}+c^{(d-logK)})$, to the diastolic data. The constants derived were a = 3.4213, b = 6.3056, c = 51.1540, d = 5.7185. This function was not used in the fitting of the Ca–ATP–Ca model to the experimental data. The broken line represents the expected systolic fraction bound calculated from the diastole fitted curve using the constants derived from the model fitting. However, the difference between the two curves was used to show the result of the model fitting in Figure 3.

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 sub units, i.e. M has two troponin sites. The predicted affinities of the first and second ATP-independent Ca^{2+} binding were 10^6 and $10^{5.4}M^{-1}$ respectively. The apparent dissociation constant for the myofibrillar Ca^{2+} binding was 580nM (in the presence of saturating phosphocreatine (PCr) and 5mM ATP) and the concentration of Ca^{2+} binding sites was $2 \times 29 = 58\mu M$. The total mobilized Ca^{2+}, $\Delta[Ca]_T$, was $44\mu M$. The value for $log(K_3 K_p)$, the ATP and subsequent Ca^{2+} binding step, was 10.6. During low stringency fitting the value of K_p rapidly approached $10^{8.5}$ and increased to very high values as the stringency was increased.

In addition, if the two ATP-independent Ca^{2+}-binding steps (K_1 and K_2) 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 $29\mu M$ and an affinity of $10^{5.7}M^{-1}$.

Comparison of the model predicted and in vitro measurements of the $[Ca^{2+}]$ dependence of the myofibrillar ATPase

Our model was constrained to fit data obtained under *in vivo* conditions, i.e. in the presence of saturating PCr and [ATP] = 5mM [22]. The *in vitro* data, 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] = 3mM. 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, Equation 4] was a minimal $13.6\mu M$ in the presence of saturating PCr and $79.9\mu M$ in the absence of PCr. The value obtained for $K_3 K_p$ depends on this apparent ATP affinity [see Equation 4]. Therefore, to reproduce the Ca^{2+} dependence of the ATPase under conditions *in vitro* we recalculated $K_3 K_p$ for the absence of PCr. In addition to the absence of PCr the data of Holroyde *et al.* [11] were obtained at 4mM $[Mg^{2+}]$ whereas under the conditions of the NMR experiments (and therefore in our model) the $[Mg^{2+}]_i$ is 1.2mM [23]. It has been shown that the apparent affinity of the ATPase for Ca^{2+} is $[Mg^{2+}]$ dependent; a five-fold increase in the $[Mg^{2+}]$ from 2 to 10mM causes a 0.38 pCa shift in the apparent K_d for Ca^{2+} of the ATPase [24]. Therefore, assuming the apparent Ca^{2+} dissociation constant has linear dependence on $[Mg^{2+}]$ over the range 1 to 10mM, we have generated values for the data of Holroyde *et al.* [11] that would be expected at 1.2mM Mg^{2+}, by adding 0.3 to their pCa values.

The data for the Ca^{2+} dependence of the myofibrillar ATPase under conditions *in vitro*, as above, is plotted in Figure 6 (page II-10) (◆) along with the Ca^{2+} dependence of the myofibrillar ATPase predicted by the Ca–ATP–Ca model (solid line) as fitted to the NMR data (Table 1) and the [ATP] dependence data of Krause and Jacobus [15].

Additional endogenous buffers

For the Ca–ATP–Ca model the predicted affinity and/or concentration of the additional endogenous buffer, K_E and $[E]_T$, are too low to make any significant contribution to Ca^{2+}-buffering. These results rule out other endogenous non-cooperative cytosolic Ca^{2+} buffers contributing to buffering of the cardiac
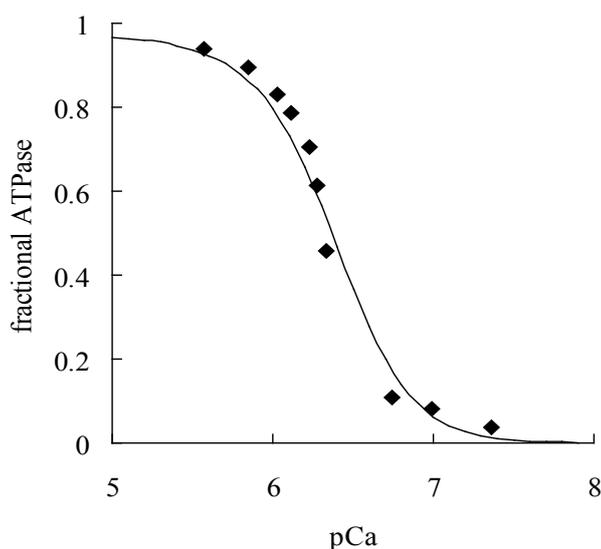
Figure 6: Model predicted values for [Ca²⁺] dependency of the myofibrillar ATPase

Comparison of the values calculated from the Ca–ATP–Ca model for the [Ca²⁺] dependence of the myofibrillar ATPase activity (solid line) and the experimental data obtained by Holroyde *et al.*(◆) [11] corrected to that expected for a [Mg²⁺] of 1.2mM (see text for details).

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 Ca²⁺ buffer concentrations as a function of their affinities that can be added using this approach.

Ca²⁺ transient. However, if the additional buffer was cooperative with two sites for Ca²⁺ with an apparent dissociation constant close to that of the myofibrils, this buffer could be included in the total binding sites of 58μM 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 if the buffering is composed of a single component or two components with similar characteristics. Solutions to the data fitting when an additional buffer with cooperative binding is included do show minimization of the sum of squares as its concentration approaches zero, albeit with a very small gradient of approach to minimum (Figure 7).

Effect of the indicator concentration on the Ca²⁺ transient

To obtain adequate S:N ratios, ¹⁹F-NMR indicators have to be loaded to concentrations of approx. 120μM, which clearly perturbs the [Ca²⁺] transient [2]. We can use the model to calculate the perturbation of the Ca²⁺ transient that would occur for any Ca²⁺ indicator at any concentration. Figure 8 shows the predicted effect of loading an indicator with a K_d value of 220nM at intra-cellular concentrations of 0–250mM. The model predicts that even at 30μM an indicator with K_d=220nM would reduce the [Ca²⁺] transient by approx. 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].

Figure 8: Effect of Ca²⁺ indicators on cardiac Ca²⁺ transients

The expected [Ca²⁺]ᵢ transients in ferret heart loaded with varying concentrations of indicator with K_d of 220nM. It was assumed that diastolic [Ca²⁺]ᵢ rises asymptotically to a raised value at high indicator concentrations such that the diastolic [Ca²⁺]ᵢ at 120μM indicator was the same as that measured experimentally (see Table 1).

Discussion

A new kinetic model of Ca²⁺-buffering in the intact heart

Previous estimates of Ca^{2+} buffering in the heart have usually assumed that it is composed of one or more buffer species each with a single site for Ca^{2+} (e.g. [7,28]). These models could not reproduce our ^{19}F-NMR data (Figure 3). However, our re-analysis of the data *in vitro* for the $[Ca^{2+}]$ dependence of the myofibrillar ATPase showed that Ca^{2+} binding to the myofibrils (the most significant source of Ca^{2+} buffering in the heart; [10]), must bind at least two Ca^{2+} ions cooperatively (see Figure 1). We used this observation as the basis for the new model of Ca^{2+} buffering in 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^{2+} from the myofibrils must be at least $50s^{-1}$ and that Ca^{2+} 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^{2+} binding to the myofibrils, the principal cytosolic Ca^{2+}-buffer. In the model, a first Ca^{2+} is bound, followed by ATP which effectively raises the affinity of a second Ca^{2+} binding from low to high. The second Ca^{2+} is then bound. Hydrolysis of the ATP (with a rate constant of $65s^{-1}$ [18]) causes the second Ca^{2+} binding site to revert to its low affinity state thereby enabling the myofibrils to relax through rapid release of Ca^{2+}.

Estimation of Ca²⁺-buffering in the intact heart

The new kinetic model of Ca^{2+}-buffering was constrained to fit our data for the ^{19}F-NMR measurement of $[Ca^{2+}]_i$ in the Langendorff-perfused ferret heart as well as the *in vitro* data 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^{2+} binding sites of $58\mu M$ with half activation of the ATPase at 580nM. Solaro *et al.* [29] estimated the concentration of high affinity Ca^{2+} specific binding sites in ventricular muscle to be $\approx 70\mu M$, which is close to the value derived from our model.

To estimate the concentration and affinities of endogenous Ca^{2+} buffers it was necessary to know how much Ca^{2+} was released into the cytoplasm during each Ca^{2+} transient. To allow for the possibility that the depolarization-induced Ca^{2+} release may vary with the increased buffering imposed by loading a Ca^{2+} indicator, a "Ca^{2+} store" with internal concentration $[Ca^{2+}]_o$, and a release dependent on the difference, $[Ca^{2+}]_o - [Ca^{2+}]_i$, was included in the

model as a mathematical device. For such a store, if the $[Ca^{2+}]_o$ is close to $[Ca^{2+}]_i$ then the driving force for Ca^{2+} release will be small and the amount released will be highly dependent on $[Ca^{2+}]_i$ and therefore on the presence of Ca^{2+} indicators if they perturb the $[Ca^{2+}]_i$. However, if the $[Ca^{2+}]_o$ is high relative to the $[Ca^{2+}]_i$ then the driving force for release will be large and the amount of Ca^{2+} released will be effectively independent of $[Ca^{2+}]_i$. In solutions to our model the amount of Ca^{2+} mobilized remained constant, $44\mu M$ for the Ca–ATP–Ca model, which suggests that there is no increase in mobilized Ca^{2+} induced by the indicator. This prediction is consistent with the recent estimation of the concentration of free Ca^{2+} in the sarcoplasmic reticulum of $\approx 700\mu M$ buffered by 14mM binding sites with $K_d \approx 640\mu M$ [30]. It is also in agreement with the study from Adachi-Akahane *et al.* [31] who showed that the amount of Ca^{2+} released from the sarcoplasmic reticulum was unaffected by increasing the cytosolic Ca^{2+} buffering up to 16mM (2mM Fura-2 plus 14mM EGTA). Estimates from the model of $40–44\mu M$ mobilized Ca^{2+} are very similar to the recent estimates of Ca^{2+} release in ferret ventricular myocytes of $30–60\mu M$ [32], but are less than those estimated in many previous studies (typically $100–130\mu M$: [7,10,33]). It is possible that the earlier studies overestimated the release of Ca^{2+} because they were assumed much higher values for Ca^{2+} buffering than were calculated in our model. It is also possible that species variations, which are known to contribute to differences in Ca^{2+}-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_d values for the four calmodulin Ca^{2+} binding sites, when measured under *in vivo* conditions, all have values well below 100nM 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 $[Ca^{2+}]_i$. The K_d for the sarcolemmal Ca^{2+} binding sites has been estimated to be 180nM [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+} re-uptake by the sarcoplasmic reticulum (SR) could be best described by a process that binds Ca^{2+} with a Hill coefficient

of 2 and is half-saturated when $[Ca^{2+}] = 500nM$ [36]. These parameters would enable the SR to make a significant contribution to Ca^{2+}-buffering during the cardiac Ca^{2+} transient. However, the SR 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 SR 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 Ca²⁺-indicators on cardiac Ca²⁺ 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_d of 220nM 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 $[Ca^{2+}]_i$, such concentrations of added indicators will also slow the transients (e.g. see [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).

The Hill coefficients of the myofibrillar ATPase and ⁴⁵Ca binding

A conventional model of cardiac myofibrillar ATPase activity, i.e. one which included a single Ca^{2+}-binding site and an ATP-binding step, was unable to reproduce our experimental data (see Figure 4). 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 cooperativity of ^{45}Ca 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 cooperativity. In the presence of ATP the species formed (MCa_2ATP) has high Ca^{2+} cooperativity but again only one Ca^{2+} per sub unit. The results in this paper introduce an unexpected mode of interaction between sub units of the myofibril to answer the question of how the apparent cooperativity 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 sub units, 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 cooperativity between ATPases or with cross bridges. The present model requires that cardiac myofibrils have a second Ca^{2+} binding site on an interacting sub unit with an apparent affinity that is modulated by ATP binding. The situation may also be viewed as the binding of ATP reduces the affinity of interacting sub units to bind the first Ca^{2+} and increases that for the second. Importantly, only one ATP is hydrolysed by the two interacting sub units.

The situation is similar in the cooperative 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].

Acknowledgements

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