The effect of Mg^{2+} on cardiac muscle function: is CaATP the substrate for priming myofibril cross-bridge formation and Ca^{2+} reuptake by the sarcoplasmic reticulum?

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Kinetics are established for the activation of the myofibril from the relaxed state [Smith, Dixon, Kirschenlohr, Grace, Metcalfe and Vandenberg (2000) Biochem. J. **346**, 393–402]. These require two troponin Ca^{2+}-binding sites, one for each myosin head, to act as a single unit in initial cross-bridge formation. This defines the first, or activating, ATPase reaction, as distinct from the further activity of the enzyme that continues when a cross-bridge to actin is already established. The pairing of myosin heads to act as one unit suggests a possible alternating mechanism for muscle action. A large positive inotropic (contraction-intensifying) effect of loading the Mg^{2+} chelator citrate, via its acetoxymethyl ester, into the heart has confirmed the competitive inhibition of the Ca^{2+} activation by Mg^{2+}, previously seen *in vitro*. In the absence of a recognized second Ca^{2+}-binding site on the myofibril, with appropriate binding properties, the bound ATP is proposed as the second activating Ca^{2+}-binding site. As ATP, free or bound to protein, can bind either Mg^{2+} or Ca^{2+}, this leads to competitive inhibition by Mg^{2+}. Published physico-chemical studies on skeletal muscle have shown that CaATP is potentially a more effective substrate than MgATP for cross-bridge formation. The above considerations allow calculation of the observed variation of fractional activation by Ca^{2+} as a function of $[Mg^{2+}]$ and in turn reveal simple Michaelis–Menten kinetics for the activation of the ATPase by sub-millimolar $[Mg^{2+}]$. Furthermore the ability of bound ATP to bind either cation, and the much better promotion of cross-bridge formation by CaATP binding, give rise to the observed variation of the Hill coefficient for Ca^{2+} activation with altered $[Mg^{2+}]$. The inclusion of CaADP within the initiating cross-bridge and replacement by MgADP during the second cycle is consistent with the observed fall in the rate of the myofibril ATPase that occurs after two phosphates are released. The similarity of the kinetics of the cardiac sarcoplasmic reticulum ATPase to those of the myofibril, in particular the positive co-operativity of both Mg^{2+} inhibition and Ca^{2+} activation, leads to the conclusion that this ATPase also has an initiation step that utilizes CaATP. The first-order activation by sub-millimolar $[Mg^{2+}]$, similar to that of the myofibril, may be explained by Mg^{2+} involvement in the phosphate-release step of the ATPase. The inhibition of both the myofibril and sarcoplasmic reticulum Ca^{2+}-transporting ATPases by Mg^{2+} offers an explanation for the specific requirement for phosphocreatine (PCr) for full activity of both enzymes *in situ* and its effect on their apparent affinities for ATP. This explanation is based on the slow diffusion of Mg^{2+} within the myofibril and on the contrast of PCr with both ATP and phosphoenolpyruvate, in that PCr does not bind Mg^{2+} under physiological conditions, whereas both the other two bind it more tightly than the products of their hydrolysis do. The switch to supply of energy by diffusion of MgATP into the myofibril when depletion of PCr raises [ATP]/[PCr] greatly, e.g. during anoxia, results in a local $[Mg^{2+}]$ increase, which inhibits the ATPase. It is possible that mechanisms similar to those described above occur in skeletal muscle but the Ca^{2+} co-operativity involved would be masked by the presence of two Ca^{2+}-binding sites on each troponin.

Key words: acetoxymethyl citrate, myofibrillar ATPase, phosphocreatine, sarcoplasmic reticulum, ATPase.

INTRODUCTION

Muscle contraction is critically dependent on $[Ca^{2+}]_i$ and ATP hydrolysis [1] (ATP refers to free unligated ATP, unless otherwise specified). The structural basis of contraction and the mechanisms by which Ca^{2+} regulates contraction and by which the energy produced by ATP hydrolysis is transduced into force development and/or movement have been the source of intense investigation for over 50 years. Muscle contraction is a complex process involving many proteins and the formation of numerous intermediates. It is therefore not surprising that even now there are still many unexplained observations about the detail of the mechanism of muscle contraction [2]. Ischaemic contractile failure of the heart is a major cause of morbidity and mortality [3]. Understanding muscle contraction and why it goes wrong, under conditions such as acute myocardial ischaemia, is therefore of major biomedical importance.

Elevations in inorganic phosphate, a fall in pH_i and decline in [phosphocreatine] ([PCr]) and [ATP] have all been implicated in ischaemic contractile failure [3]. Elevations in intracellular free magnesium concentrations, $[Mg^{2+}]_i$, occur during ischaemic/hypoxic episodes in cardiac muscle. Previous work in skinned fibres [4,5] and isolated cardiac myocytes [6] has shown that increases in $[Mg^{2+}]_i$ cause an inhibition of myofibril contraction and ATPase activity and so could also contribute to ischaemic contractile failure [7]. Similarly, increases in $[Mg^{2+}]_i$ have been shown to inhibit the myofibril ATPase (EC 3.6.4.1 [4,8]). $[Mg^{2+}]_i$ is also known to be an important regulator of many other

Abbreviations used: LVDP, left-ventricular developed pressure; AM, acetoxymethyl; SR, sarcoplasmic reticulum; S-1, myofibril subfragment 1; 5FBAPTA, 1,2-bis-(2-amino-5-fluorophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetra-acetic acid; PCr, phosphocreatine; AM citrate, Tris(acetoxymethyl) citrate.

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functions in cardiac myocytes, including Ca^{2+} reuptake by the sarcoplasmic reticulum (SR) Ca^{2+}-transporting ATPase (EC 3.6.3.8 [9]) and many plasma-membrane ion channels (e.g. [9a]). The variety of Ca^{2+}- and Mg^{2+}-binding sites identified in muscle makes interpretation of the underlying mechanisms a mammoth task in which little progress has been made.

Numerous reports in the literature are consistent with the suggestion that there must be two calcium-binding sites on cardiac myofibrils that act co-operatively to activate the myofibril ATPase (reviewed in our previous paper, [10]). This is consistent with the presence of two un-cross-linked myosin heads in the actomyosin myofibril subfragment 1 (S-1) complex in the relaxed condition. This state is physically different from the situation where one of the myosin units is already cross-linked to the actin filament and subsequent ATPase activity is possible via participation of the second myosin head. This and the presence of cross-bridges to both myosin heads in the rigor state [11,12] strongly suggest that the contractile functional unit consists of the pair of myosin heads operating alternately. To date, however, there has only been one myofibril Ca^{2+}-binding site, with an appropriate Ca^{2+} affinity, identified in cardiac myofibrils, i.e. the Ca^{2+}-binding site in troponin C [13]. This apparent discrepancy, coupled with the known inhibition of contraction by rises in $[Mg^{2+}]_i$, has led us to hypothesize that the substrates for recruitment of the myofibril units to form cross-bridges are Ca^{2+} (bound to troponin C) and CaATP. Having CaATP as a substrate provides the second Ca^{2+} binding site, and could also explain inhibition by Mg^{2+}, in terms of competing for binding to free ATP. The aim of this paper was to formulate a model of cardiac contractile activity incorporating Ca^{2+} and CaATP as the apparent substrates for myofibril activation. (If an enzyme, E, requires the binding of two components, A and B, for activity, then at equilibrium it does not matter whether E binds A, B or AB first in forming the active complex EAB. We use the term effective substrate for the species AB whichever of these pathways predominates.) We have tested this model by loading hearts with a Mg^{2+} chelator to reduce $[Mg^{2+}]_i$, thus confirming the *in vitro* observations of the effect of $[Mg^{2+}]$. Furthermore, we noted that there are many similarities between the effect of $[Mg^{2+}]_i$ on SR Ca^{2+} uptake and contractile ATPase activity (see e.g. [7]) and therefore investigated whether the same Ca^{2+}/CaATP model could reproduce the effects of Mg^{2+} on SR Ca^{2+} reuptake.

It should be noted that the citations and data in this paper concern isometric contraction, or initial rates of unconstrained myofibril ATPase, or the rate of SR Ca^{2+}-transporting ATPase in the absence of a gradient of $[Ca^{2+}]$. In all these cases the muscle performs minimal external work. During isometric contraction no longitudinal movement of the cross-bridged units along the actin filaments can occur. Unconstrained myofibrils fully contract with minimal ATP usage, as there is no constraint to longitudinal movement. It is therefore possible that the schemes presented are applicable only to 'activation' of the enzymes and hence 'initiation' of the contractile ATPase cycle. This does not exclude MgATP as the preferred substrate for subsequent energy consumption and consequent work, e.g. via cross-bridge cycling. The concerted action of the pairs of head groups has not been considered previously [1,14].

MATERIALS AND METHODS

Synthesis of the acetoxymethyl (AM, Ac-O-CH_2-) ester of citric acid

The AM ester of citric acid was prepared by essentially the same method used for one of the non-toxic isomers of fluorocitrate [15]. Briefly citric acid was suspended in dichloromethane and treated with an excess (4 mol/mol) of ethyl(di-isopropyl)amine and stirred until the solid dissolved. Water was removed by the addition of toluene and removal of the solvents *in vacuo*. Complete dryness was assured by twice dissolving in dichloromethane and evaporating with toluene *in vacuo*. The dried oil was dissolved in dichloromethane and treated with ethyl(di-isopropyl)amine (2 mol/mol), cooled in iced water and stirred during the addition of bromomethyl acetate (3 mol/mol). After 3 h, TLC (silica gel, developed in 40 % ethyl acetate in toluene, visualized by iodine staining) revealed two major products. The solvent was removed *in vacuo*, the gummy solid triturated with a little ethyl acetate, the slurry diluted with toluene (4 vol.) and filtered. The filtrate was subjected to column chromatography (silica 60, 100 g/g of citric acid) eluting with ethyl acetate in toluene (20–80 %) and the more polar of the two main components collected and evaporated to dryness *in vacuo* (water bath below 40 °C). The tertiary hydroxy group of the citrate renders the AM ester inherently unstable, so it was stored at −80 °C and checked by TLC before use.

Heart perfusion

Hearts were excised rapidly from male ferrets ($\approx$ 6 months old) weighing < 1.5 kg, which had been anaesthetized with sodium pentobarbitone (250 $mg \cdot kg^{-1}$, intraperitoneally) and heparinized (2000 i.u., intraperitoneally). The hearts were Langendorff-perfused at a constant flow rate of 5 $ml \cdot min^{-1} \cdot (g\ wet\ weight)^{-1}$ and monitored as previously described [16,17].

Loading the hearts with buffers

The 1,2-bis-(2-amino-5-fluorophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetra-acetic acid (5FBAPTA) and citrate, used to buffer Ca^{2+} and Mg^{2+} respectively, were loaded as cell-permeant AM ester derivatives [15,18]. 5FBAPTA was made up as a 50 mM stock solution in DMSO and loaded into the hearts at a constant rate of 250 μl/h by infusion into the lines carrying the perfusate via a syringe pump, downstream of the bubble trap and filter systems, as previously described [15,16,18]. Tris(acetoxymethyl) citrate (AM citrate) was loaded as described for 5FBAPTA-AM but using a 400 mM stock solution of AM citrate, or by adding the AM citrate directly to perfusion solutions at a concentration of 1 mM.

Oxalate-supported Ca^{2+} uptake

Homogenates were prepared as described by Vetter and Will [19]. Briefly, powdered heart tissue was homogenized at 2 °C in high-salt histidine buffer with EGTA and clarified by moderate centrifugation. The crude membranes were sedimented for 45 min at 148 000 ***g*** at 2 °C before being rehomogenized and sedimented. After an additional wash with 0.2 mM dithiothreitol/0.1 mM PMSF/10 mM histidine (pH 7.4), crude membranes were suspended in 0.25 M sucrose/10 mM histidine (pH 7.4) at a final protein concentration of 20 mg/ml. This homogenate was used within 10 min for the measurement of oxalate-supported Ca^{2+} uptake.

Control oxalate-supported Ca^{2+} uptake into SR vesicles was measured as described previously [20] at 37 °C, pH 7.0, with 6 mM PCr, 333 nM free Ca^{2+} and 3.63 mM MgATP. After pre-incubation of the reaction mixture for 2 min in the absence of homogenate, the Ca^{2+} uptake was started by the addition of 80 μg of homogenate protein per ml of assay buffer. Samples were taken at various time intervals, filtered, washed with EGTA buffer and radioactivity associated with dried filters was de-

termined by liquid scintillation counting. Transport rates were calculated by the linear regression of the data points. The procedure allowed for Ca^{2+} uptake into SR vesicles only [21].

The Ca^{2+} uptake assay into SR vesicles was repeated for a range of $[Mg^{2+}]_{free}$, with the required total ATP, at the same fixed [MgATP] and $[Ca^{2+}]_{free}$ as in the control uptake.

Miscellaneous

Data were fitted using Microsoft Excel Solver as described by Smith et al. [10]. Free ion concentrations were calculated using the Fabiato programme [22] as described by Vetter and Rupp [23]. Protein was determined by the method of Lowry et al. [24] using ovalbumin as a standard. All the chemicals used here were from Sigma unless otherwise stated.

RESULTS AND DISCUSSION

Contractile strength and $[Mg^{2+}]_i$

There has been no means of readily altering the cytoplasmic magnesium concentration, $[Mg^{2+}]_i$, other than by patch-clamp experiments [9a] or prolonged depletion of Mg^{2+} in the medium [26]. We therefore sought a method to achieve this. The citrate ion is a chelator of intermediate strength for most polyvalent cations and this with its highest pK of 5.69 makes it an excellent buffer for polyvalent-cation titrations, since hydrons will not compete with binding of other cations. The affinities of citrate for Mg^{2+} (log$K$, 3.37) and Ca^{2+} (log$K$, 3.50) [27] ensure that it will bind the former but not the latter under normal cytoplasmic conditions. A slightly weaker chelator, one of the three non-toxic isomers of fluorocitrate [28], has been used as a ^{19}F-NMR indicator to measure $[Mg^{2+}]_i$ in the ferret heart [15]. During these experiments a small but consistent rise in contractile strength, left-ventricular developed pressure (LVDP), was observed after loading the AM ester. We therefore prepared this ester of citric acid, which could be loaded into the cytoplasm where acetylases would release citrate and thus reduce the $[Mg^{2+}]_i$. Perfusion of the ferret heart with AM citrate, under the same conditions as used for the fluorocitrate [15], resulted in the same very small but consistent increase in LVDP. We then reasoned that the Langendorff-perfused heart was already working at near its maximum cyclical contractility and to observe a larger increase in contraction the systolic $[Ca^{2+}]_i$ had to be reduced. Therefore the experiment was repeated, but before the loading with AM citrate, the heart was equilibrated with a medium of sufficiently reduced $[Ca^{2+}]$ to give about 50 % of the maximum LVDP. The continuously recorded pressure trace from this experiment is shown in Figure 1.

Loading hearts with AM citrate resulted in an initial decrease in contraction, presumably due to the release of H^+ into the cytoplasm by the esterase action (six H^+ per AM citrate). Subsequently there is a sustained increase in LVDP (to 161 % of the initial value, see Figure 1). The maximum LVDP observed in hearts perfused with 0.9 mM Ca^{2+} and loaded with citrate was always higher than that recorded from the same hearts perfused with 1.8 mM Ca^{2+} before loading AM citrate ($n = 3$). The loading of citrate into the cytosol will have minimal effects on the bioenergetics, as it is always present in concentrations sufficient to saturate the enzymes of the citric acid cycle. We reason that the increased contraction is due solely to the increased response of the myofibrillar ATPase to $[Ca^{2+}]_i$ in the reduced $[Mg^{2+}]_i$ (see below). The final very slow decline in the LVDP reflects the slow flux of both Mg^{2+} and citrate through the plasma membrane.

The myofibrillar sites for binding bivalent cations

By correlation of the Ca^{2+}-activation profile and the measured Ca^{2+} affinities of intact and fractionated myofibrils, Holroyde et al. [29] identified the troponin type II Ca^{2+}-binding sites, two per troponin molecule in skeletal muscle and one in cardiac muscle, as those solely responsible for activation of the myofibrils by $[Ca^{2+}]$. The affinity for Ca^{2+} of these type II sites does not vary over the physiological range of $[Mg^{2+}]$ in isolated troponin [29] and probably does not do so in the intact myofibril lacking ATP [29–32]. All the remaining known muscle-protein Ca^{2+}-binding sites, some with the ability to bind Mg^{2+} competitively, e.g. the type I sites on troponin, have binding kinetics for these cations that preclude a direct effect of Mg^{2+} on the Ca^{2+} activation of the ATPase (see below). Thus all measured Ca^{2+} binding that correlates with activation is unaffected by $[Mg^{2+}]$. These conclusions, however, are based on static experiments performed in the absence of ATP. The Hill coefficient, but not the amount, of Ca^{2+} binding to myofibrils in the range activating the ATPase is increased by the presence of ATP [31]. The ATP-induced cooperativity of Ca^{2+} binding must mean that ATP binding creates, or converts a low-affinity site into, a high-affinity site for calcium. Thus it is possible that the presence of ATP similarly introduces possibilities for Mg^{2+} to bind in competition with Ca^{2+}. Only one study exists of the effect of $[Mg^{2+}]$ on the Ca^{2+} binding of myofibrils in the presence of ATP [30], but unfortunately the error limits on the data are too wide to permit definitive conclusions. In summary, no functional Ca^{2+}/Mg^{2+} competitive binding has been observed in the $[Ca^{2+}]$ activation range in the absence of ATP, but in the presence of ATP it has been neither observed nor ruled out.

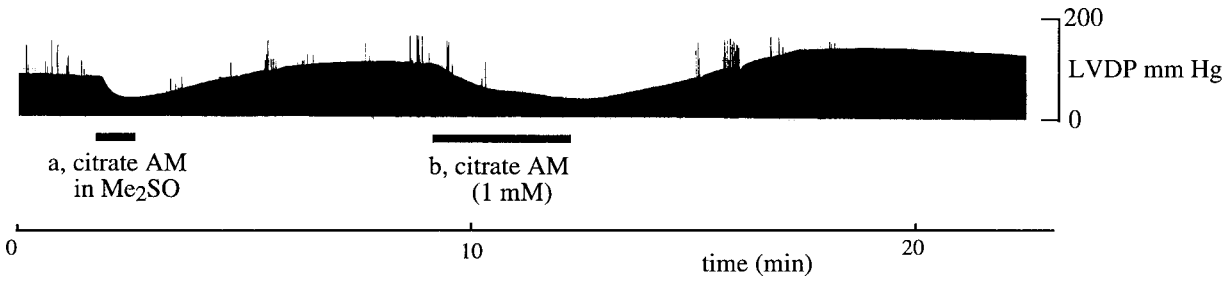
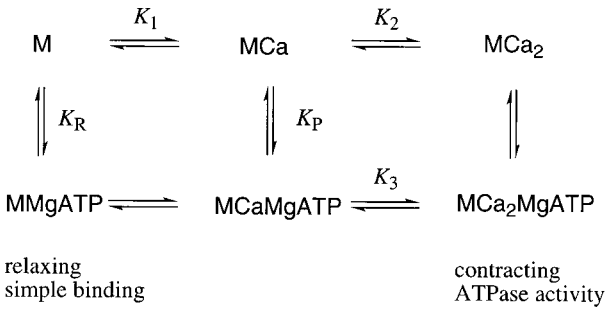


Figure 1 Changes in LVDP during loading of AM citrate

LVDP recorded from an isolated ferret heart (6 g) perfused with 0.9 mM Ca^{2+} at 30 ml/min during infusion of (a) AM citrate (400 mM in DMSO), added to the perfusate at 30 μl/min and (b) AM citrate (1 mM) added directly to the perfusate.



Scheme 1 Simple model for activation of myofibrils [10]

The Ca^{2+}-free myofibril functional unit (M) binds the first Ca^{2+} ion with affinity K_1, to form MCa, which binds MgATP with affinity K_P. Whereas MCa binds a second Ca^{2+} ion with fairly low affinity, K_2, MCaMgATP binds a second Ca^{2+} with much greater affinity, K_3. Also included is the Ca^{2+}-independent ATP binding (affinity K_R), which has been equated with the 'loose-bound ATP state' and results in relaxation of the protein.

Effects of [Mg^{2+}] on the cardiac myofibril

The following argument demonstrates that there is an inconsistency between the measured Ca^{2+} and Mg^{2+} binding summarized above and the reported effects of these ions on function. Two sets of studies published in the 1970s [33–35] indicate that Mg^{2+} has a complex effect on the Ca^{2+}-stimulated activity of myofibrils. The data of Solaro and Shiner [33] on the ATPase of skeletal muscle appear to reflect a direct competition of Mg^{2+} for the activation by Ca^{2+}, accompanied by a small decrease in the enzyme V_{max} with increasing [Mg^{2+}], both occurring over the whole of the [Mg^{2+}] range of 0.04–5.0 mM. The cardiac-muscle ATPase data are less straightforward and are best described by a pattern similar to the skeletal-muscle data but with the addition of a large increase of enzyme V_{max} as [Mg^{2+}] increases in the lower part of the range, below 1 mM. Since there is no measured competition by Mg^{2+} in the physiological range at the recognized activating Ca^{2+} sites of troponin, which are spatially well separated from the myosin ATPase site, and the associated affinities for Ca^{2+} and Mg^{2+} are not altered by this interaction, we can draw some conclusions. We can assume that the competitive Ca^{2+} and Mg^{2+} binding measured in the absence of ATP [33] is at structural sites, and would thus affect the ATPase reaction by altering the accessibility and/or flexibility of the catalytic site. This may change the enzyme V_{max}, but will not change the fraction of activating Ca^{2+} sites that are occupied. We can remove such effects by plotting activity against pCa at different pMg values, with each rate normalized to fractional rates of the V_{max} observed at that pMg. What remains will show any direct effects of [Mg^{2+}] on the Ca^{2+} control of the ATPase. Solaro and Shiner [33] (see their Figure 2) made this transformation and showed that Mg^{2+} inhibits the myofibril ATPase contractile activation by Ca^{2+} in a clearly competitive manner. The above directly observed effect of Mg^{2+} on the Ca^{2+} stimulation of the ATPase is strongly corroborated by the isometric contraction data of Donaldson et al. [35]. No satisfactory explanation of this phenomenon has been advanced and the effects of Mg^{2+} are still often explained only as resulting from undefined structural changes.

A candidate for Mg^{2+}-inhibitable Ca^{2+} binding

In an early study of muscle activation by Ca^{2+}, Ashley and Moisescu [14] concluded, "Alternatively, there might be one Ca^{2+} bound on troponin and one on the myosin filament, the site on myosin being induced *in vivo*". In a recent study of Ca^{2+} buffering in the heart, Smith et al. [10] proposed a possible extra Ca^{2+} binding that exists under *in vivo* conditions. Their kinetic considerations led to the conclusion that such an additional Ca^{2+}-binding site on the myofibril is formed when ATP binds and must be filled for activation. The scheme for myofibril activation by Ca^{2+} that they derived, the Ca-ATP-Ca model, was shown in their Scheme 3 [10]. Although this did not show Mg, since they used [MgATP] as the substrate concentration, their scheme was effectively Scheme 1.

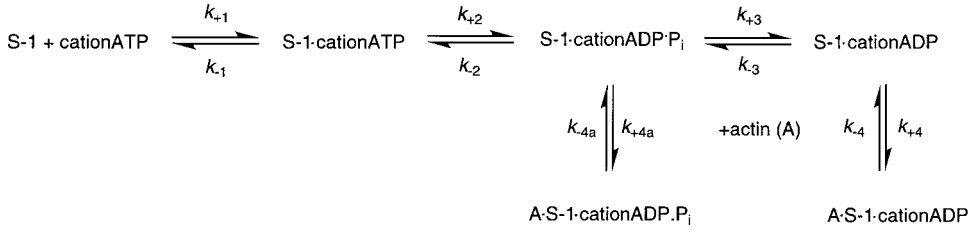
In the study of Smith et al. [10] the predicted ATPase activation, derived from what was essentially a Ca^{2+}-binding study, was correlated with the Ca^{2+} activation of the ATPase previously measured *in vitro* [29]. The data sets compared were obtained at different [Mg^{2+}] values, necessitating an empirically derived pCa offset correction to one data set. If, however, the formation of the active species, via the equilibria assigned the association constants K_P and K_3, was competitively inhibited by Mg^{2+}, then the form of the normalized [Mg^{2+}] dependency of the Ca^{2+} activation (at constant [MgATP]) [33] is readily obtained (see below). Thus for the '*in vivo*' of Ashley and Moisescu [14] we may now substitute 'in conditions more closely resembling those *in vivo*, i.e. in the presence of ATP'.

A possible model of competitive Ca^{2+}/Mg^{2+} activation of the myofibril ATPase

Physico-chemical evidence of CaATP as a possible substrate: evidence from skeletal muscle

Polosukhina et al. [36], using transient electric birefringence, obtained evidence that myosin cross-bridges are more compact when CaATP, rather than MgATP, is hydrolysed.

The activation of cardiac myofibrils by low levels of [Mg^{2+}], of which the origin and independence from the Ca^{2+} activation are



Scheme 2 The steps leading to cross-bridge formation in the absence of troponin

S-1 may be regarded as the myofibril (M) without either the actin (A) or the Ca^{2+}-mediated control by troponin. The cation is Ca^{2+} or Mg^{2+} ligated to the nucleotide. Steps 1–4 and 4a are indicated by the subscripts used for the forward and backward rate constants, e.g. k_{+1} and k_{-1} respectively for step 1.

Table 1 Physico-chemical constants of skeletal muscle for Scheme 2

Rate constants compiled from Polosukhina et al. [36] and Peyser et al. [38], who used analogues of P_i to gain insight into the steps involved in the ATPase reaction and cross-bridge formation. The rate constants for step 3 are for the reaction where the analogue replaces P_i. These are obtained by measurement of the rate and equilibrium limit of deactivation of the ATPase by the inhibitory analogue. The inhibitor tetrafluoroberyllate(2−) is an equilibrium mixture prepared by mixing 0.2 mM Be^{2+} and 5 mM F^- in the reaction buffer. Note, the equilibrium constants of actin binding are in Napierian logarithms as cited.

Step	Constant	Ca^{2+}	Mg^{2+}	P_i analogue	Reference
1–4	K_m (μM) (m^{2+}ATP)	1.6	19.2	P_i	[38]
1–4	V_{max} (s^{-1})	7.1	14.7	P_i	[38]
1–3	V_{max} (s^{-1})	0.317	0.042	P_i	[38]
−3	k_{-3} (s^{-1})	Too fast	0.043	BeF_4^{2-}	[38]
+3	k_3 (s^{-1})	2.0E−2	0.145E−5	BeF_4^{2-}	[38]
−3	k_{-3} (s^{-1})	1.1E−4	1.7E−2	Vanadate	[36]
+3	k_{+3} (s^{-1})	2.0E−5	3.1E−5	Vanadate	[36]
+4	ln$K$	17.1	14.1	Vanadate	[36]
+4	z^Az^M (esu^2)	−9.7	−3.6	Vanadate	[36]
+4a	ln$K$	7.6	8.7	Vanadate	[36]
+4a	z^Az^M (esu^2)	−12.2	−6.4	Vanadate	[36]

shown below, will mask comparative studies of Ca^{2+}/Mg^{2+} activation of the ATPase. For this reason the studies in this section relate to skeletal muscle, where the activation by low levels of [Mg^{2+}] is less apparent. Scheme 2 shows the generally accepted mechanism of cross-bridge formation in the absence of the Ca^{2+}-regulatory troponin. A major difference between cardiac and skeletal myofibril cation binding is also removed along with the troponin. The physico-chemical constants of skeletal muscle for Scheme 2 are summarized in Table 1.

In Scheme 2, for the overall reaction in the presence of actin (A), steps 1–4, the K_m for the nucleotide is more than one order of magnitude lower when the ligating cation is Ca^{2+} than it is with Mg^{2+}. Although these values are from a stopped assay, and reflect the steady-state condition where cross-bridge cycling can occur, there is a clear preference for the binding of CaATP. This K_m reflects the affinity of the myosin S-1 fragment for the ligated nucleotide.

For the rates of production of P_i, as before while actin is present (V_{max} in steps 1–4), there is only a 2-fold difference in favour of Mg^{2+}. However, this is again from steady-state measurement where cross-links are present, and cycling, during the bulk of the P_i formation. The relative rates (V_{max}) in the absence of actin (steps 1–3) are a better reflection of the initiation reaction, where no actin cross-link has yet been formed. In this case there is almost an order of magnitude in favour of CaATP as substrate. An alternative view is that actin is required for the rapid hydrolysis of MgATP, whereas it is not for that of CaATP. Both the K_m and V_{max} require re-investigation by rapid measurement of initial rates to avoid inclusion of subsequent MgATP usage during cycling, now possible using a fluorescent P_i-binding protein [37].

For the P_i release in the absence of actin (step 3), the studies by Peyser et al. [38] and Polosukhina et al. [36] agree on the relative magnitude of the equilibrium constants (k_{+1}/k_{-1}) when comparing Ca^{2+} with Mg^{2+}. These both indicate that the forward reaction, dissociation of P_i, is greatly more favoured over association for the S-1·CaADP·P_i complex than that with Mg^{2+}. However, the rates reported for the forwards and backwards reactions for the different analogues are not consistent. This may be related to the difference between the analogues, the tetrafluoroberyllate binding weakly but vanadate more strongly than P_i itself.

At first sight the cross-bridge formation (step 4) would also appear to be much favoured for S-1·CaADP (ln$K$ = 17.1 against 14.1). This requires further consideration in that the measures of electric dipole interaction (z^Az^M, the value of the product of the charge at the surface of actin interacting with the myosin, z^A, and the charge at the surface of the myosin interacting with the actin, z^M, as determined from the ionic strength variation of the affinity of that interaction), derived from ionic strength effects, are very different in the two cases. The difference is large enough to reflect double versus single myosin attachment to the actin. Whatever the electrostatic constants reflect, the cross-bridges form more easily when CaADP is present than with MgADP alone. The alternative cross-bridge formation, actin binding without P_i loss (step 4a), is clearly not as favoured as the sequential steps 3 and 4.

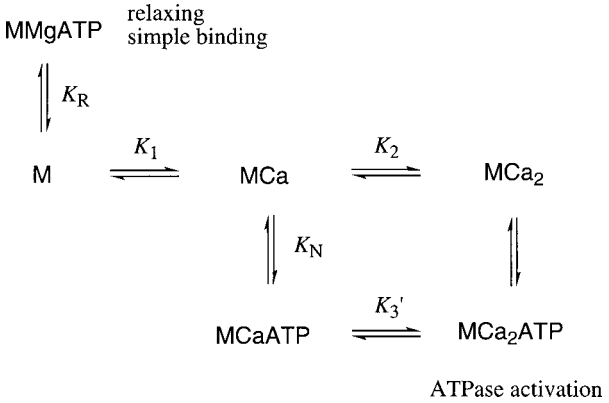
If the same holds for the cardiac myofibril, we may conclude that all the steps that lead to formation of a new cross-bridge, and hence those that follow activation by calcium binding to troponin, are favoured by the ligation of ATP by Ca^{2+}.

CaATP and MgATP as substrates of the cardiac myofibril ATPase

That MgATP is the substrate for an ATPase is a general assumption made by authors throughout the literature, a notable exception in the muscle field being some Russian groups who use CaATP as the substrate [39]. The kinetics used by Smith et al. [10] can be presented just as well by replacing the concentration of MgATP, in the generation of the active species, with that of free ATP. Inhibition by Mg^{2+} occurs in the concentration range above the dissociation constant of MgATP, where [ATP^{4-}] approaches inverse proportionality to [Mg^{2+}]. Thus the simplest modified scheme that can generate Mg^{2+} inhibition of Ca^{2+} activation is shown in Scheme 3.

Simple calculation of non-co-operative Mg^{2+} inhibition

The predictive strength of the model (Scheme 3) can be demonstrated as follows by direct calculation of the effect of variations



Scheme 3 Activation of the myofibrils

Scheme for activation of the myofibrils, assuming that free ATP, rather than MgATP, can combine with the ATPase and including the MgATP-dependent relaxation step. The constants shown are molecular constants, so that, for example, K_1 is the association constant for the first Ca^{2+} to bind, in whatever way it is distributed among sites. The free ATP is calculated from the concentration of MgATP (which approximates to the total ATP concentrations under the conditions where Mg^{2+} is inhibitory) and the known concentration of Mg^{2+} and its affinity for ATP. Then K_P, the affinity constant of MCa for MgATP in Scheme 1, is replaced by K_N, the affinity constant of MCa for free ATP. K_3' is the binding of the second, activating, Ca^{2+}.

in $[Mg^{2+}]$ on the Ca^{2+} activation of the myofibrillar ATPase, without resort to further data fitting. For the new Ca-ATP-Ca model with fixed high Ca^{2+} co-operativity (Scheme 3) all the affinity constants are the same as those found for the ferret heart by Smith et al. [10] (Scheme 1), except for the product of affinity values, $K_N K_3'$. The combined association constant for binding both Ca^{2+} and MgATP to MCa is there defined as $K_3 K_P$, and the value that fitted the data best was $10^{10.6}$ M^{-2}. If we assume that the active species is MCa_2ATP (as shown in Scheme 3) we can equate the corresponding association constant for the binding of ATP and Ca^{2+}, i.e. $K_N K_3'$, with the previous value when that value is multiplied by $[Mg^{2+}]K_{MgATP}$. Since the apparent affinity of ATP for Mg^{2+} (at the cytoplasmic pH $\approx$ 7.2 [40]) is $10^{4.2}$ M^{-1} [41,42] and the cytoplasmic $[Mg^{2+}]$ is 1.2 mM [15], the value of $K_N K_3'$ is $10^{11.8}$ M^{-2}. The change in apparent activating substrate from MgATP used by Smith et al. [10] to free ATP^{4-} removes the need to introduce the empirical correction for the different $[Mg^{2+}]$ levels used in that study.

We then applied the model in Scheme 3 to generate the expected normalized $[Ca^{2+}]$ activation curves for the myofibril ATPase at each of the $[Mg^{2+}]$ levels used by Solaro and Shiner [33]. A pCa shift of about 1.2 was required to compensate for experimental differences (see below). The agreement found was reasonable, but clearly not perfect.

Co-operative inhibition by Mg^{2+}

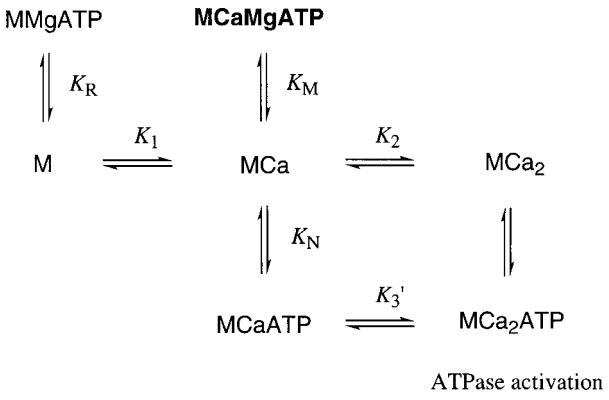
If ATP binding to the myofibril creates sites for Mg^{2+}, then Mg^{2+} can inhibit in two ways other than lowering of $[ATP]_{free}$. They are (i) the formation of MMgATP in competition with formation of MCaATP, and (ii) the formation of MCaMgATP in competition with the formation of the active species MCa_2ATP, as shown in Scheme 4.

Thus, depending on relative affinities and conditions, e.g. a sufficiently low $[ATP]_{free}$ so that MCaMgATP becomes appreciable, the Mg^{2+} inhibition, and consequently the Ca^{2+} activation, may exhibit variable degrees of positive co-operativity depending on the relative concentrations of these ions.

The solutions found previously for the Ca-ATP-Ca model (Scheme 1) imposed high Ca^{2+} co-operativity, i.e. high affinity ($>10^8$ M^{-1}) for the ATP-dependent Ca^{2+} binding (K_3) [10]. Under the *in vivo* conditions of $[Mg^{2+}] = 1.2$ mM and $[ATP]_{total} >$ 5 mM, there cannot be an appreciable concentration of a complex that contains only one calcium ion, such as MMgCaATP.

The experimental data of Solaro and Shiner [33] (their Figure 2B) suggest less-steep lines than those calculated from Scheme 3, and hence Hill coefficients substantially below 2. This is more evident in the related data published on cardiac myofibril isometric contraction, where, in the absence of a pronounced biphasic response, the maximum force is easily defined [35]. Here the Hill coefficients of Ca^{2+} activation were estimated by fitting the Hill equation to the data obtained at four $[Mg^{2+}]$ levels and are shown, with pCa values for half maximal activation at the set pMg value, in Table 2. The inactive, non-cross-bridged, species MCaMgATP in Scheme 4 will increase as the [ATP] decreases at higher $[Mg^{2+}]$ leading to a lowering of the Hill coefficient of Ca^{2+} activation in the manner observed in Table 2.

The ATPase data of Solaro and Shiner [33] (their Figure 2B) are normalized to the highest experimental value at each value of $[Mg^{2+}]$. Since the dependence on $[Mg^{2+}]$ is biphasic, so that inhibition starts before activation is complete, the true maximum for the activation, i.e. the constant that should be used for normalization, will clearly be higher than the maximum experimentally determined value. Hence to obtain more realistic values of the normalization factors we have allowed the experimental values to determine the 'true' normalization by dividing each data set (from Figure 2B in reference [33]) with a renormalization factor. Using this method we have applied Scheme 4 to the data of Solaro and Shiner [33] and obtained a least-squares fitting (Figure 2). Because this data fitting involves



Scheme 4 Activation of the myofibril (or SR) Ca^{2+}-ATPase

The scheme for the myofibril is shown, that for the SR ATPase may be obtained by replacing M with E, as is generally used for that system. This Scheme is modified from Scheme 3 to include the inactive non-cross-linked species, MCaMgATP, which is highlighted in bold. K_M is the affinity for MgATP binding to the un-cross-linked myofibrils with Ca^{2+} bound to troponin.

Table 2 Variation with pMg of the pCa (half activation) and Hill coefficient for Ca^{2+} activation of isometric force development in skinned cardiac-muscle fibres (from [35])

pMg ...	4.3	3.0	2.3	2.0
pCa (half activation)	5.56	5.06	4.59	4.37
Hill coefficient (*h*)	2.92	1.89	1.86	1.62

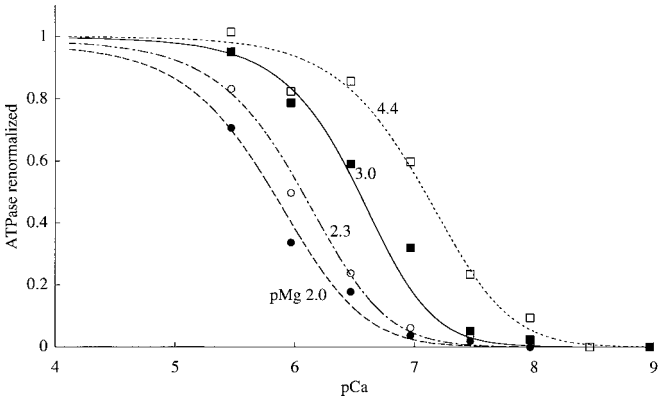


Figure 2 Variation with $[Mg^{2+}]$ of the Ca^{2+} activation of the ATPase at 30 °C (renormalized)

Our previous work, from which kinetic constants were taken, was done at 30 °C. The data are taken from Figure 2(B) of [33] and have been renormalized to the calculated V_{max} values; □, pMg = 4.4; ■, pMg = 3.0; ○, pMg = 2.3; ●, pMg = 2.0. The data were obtained at 25 °C and used an older value of 10.4 for the log of the association constant for Ca^{2+} with EGTA. The curves shown are least-squares fits to the data based on Scheme 4, allowing the values of K_N and K_M to vary along with the pCa offset and renormalization factor.

Table 3 The constants used and those derived in fitting Scheme 4 to the renormalized data for cardiac ATPase (corrected to 30 °C), as shown in Figure 2

pCa offset	log K_R	log K_1	log K_2	log K_M	log K_3'	log K_N
0.96	3.0	6.0	5.4	3.7	7.2	4.6

Table 4 Variation with pMg of the calculated pCa (half activation) and Hill coefficient for Ca^{2+} activation of cardiac myofibrillar ATPase at 30 °C (renormalized from [33]), as shown in Figure 2

pMg ...	4.4	3	2.3	2
pCa (half activation)	6.10	5.63	5.03	4.80
Hill coefficient (h)	1.13	1.07	1.18	1.13
Renormalization factor	0.99	1.05	1.20	1.41
V_{max} (normalization)	0.0137	0.0278	–	0.0288

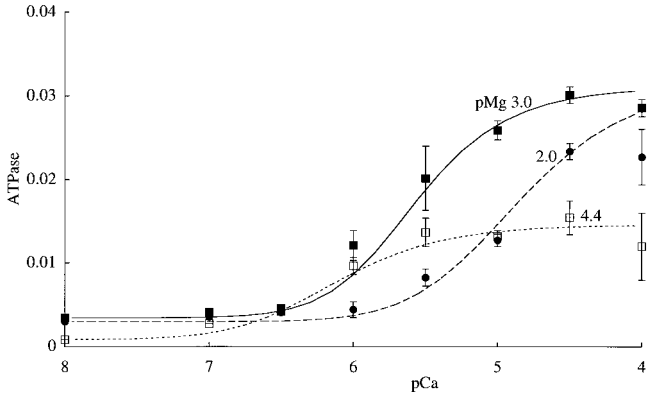


Figure 3 Variation with $[Mg^{2+}]$ and $[Ca^{2+}]$ of the myofibril ATPase at 25 °C

The theoretical curves are taken from Figure 2 with reversed pCa correction to obtain fits for 25 °C, multiplied by the equation for first-order activation by Mg^{2+} (eqn 1) and then increased by the background ATPase rates at pCa = 8 to match the conditions used in [33]. The data points, obtained from Figure 1(B) of [33], are shown for comparison. The reason why all three points at pCa 4 are below the corresponding curves is not clear. This is not common to all myofibril ATPase studies and may be related to the near saturation of EGTA with Ca^{2+} (> 99%) at pCa = 4.

new variables we have also applied the ATP apparent affinity constraint as used previously [10]. The values found for K_N and K_M and the pCa offset are given in Table 3 along with the constants used in the fitting procedure.

The renormalization factor in all cases is well below 2 (Table 4) This indicates that more than half of the competitive Mg^{2+} effect had been titrated over each pCa range, and hence that the extrapolation to the true value of V_{max} had been reliable. The 0.96 pCa offset found is reduced from the 1.2 required above, and is not far from the expected 0.8 derived from 0.4 for the corrected EGTA affinity and a consensus of around 0.4 for a 5 °C temperature shift taken from the literature. However, as expected from the wide range of conditions and species used, there is little consistency between the reported temperature coefficients of myofibril activation by Ca^{2+} [43–46].

The normalization factors (the values of V_{max}) fit first-order activation by Mg^{2+}

Analysis of the simple Mg^{2+} dependence of the activation of the ATPase is possible at saturating $[Ca^{2+}]$, i.e. in the absence of Ca^{2+}/Mg^{2+} competition. Although there are only three sets of data, and hence three values of V_{max}, at saturating $[Ca^{2+}]$ (see the corrected normalization factors, Table 4), these values are found to fit accurately to a first-order equation for activation by Mg^{2+} with an affinity of $10^{4.35}$ M^{-1}. We may numerically obtain the theoretical un-normalized curves by multiplying the theoretical values in Figure 2 by this first-order equation for activation by Mg^{2+}. This is shown in Figure 3, which is plotted against decreasing pCa and with reversed pCa offset so that it is directly comparable with Figure 1(B) of Solaro and Shiner [33], whose data points are included for comparison.

Hence the equation that best describes the kinetics of the myofibril ATPase is

$$v = V_{max} \cdot [MCa_2ATP] \cdot K_m \cdot [Mg^{2+}]/(1 + K_m \cdot [Mg^{2+}]) \quad (1)$$

This form of kinetic equation, where $[MCa_2ATP]$ is also a function of $[Mg^{2+}]$, can be theoretically derived when K_m is both a group and a molecular constant, i.e. the activation binding of Mg^{2+} only happens last in the reaction sequence or is first and obligatory for the remainder to proceed. This function and the K_m that emerged above for calculating the Mg^{2+} activation are the same as those found for the SR ATPase using a theoretical approach (see below, eqn. 4).

PCr and the myofibril

Effect of PCr on the ATPase

The myofibril consists of very tightly packed proteins. He et al. [37] demonstrated that a small molecule (NADH) diffused only slowly out of the myofibril, with a $t_{1/2}$ of 3–10 s in skinned fibres. Although smaller, the Mg^{2+} ion would be expected to have a similar diffusion rate, or may be slower because of the presence of many immobilized negative charges on the high concentration of protein.

Fibrous muscles have a complex structure of SR that enables them to overcome diffusion limits to the $[Ca^{2+}]_i$ transient that could arise within the myofibril. No such physical structure is available to ensure the sufficient supply of high-energy phosphate to the myofibrils, which is achieved predominantly by diffusion from the more distant mitochondria. In general, muscles use PCr and creatine kinase to ensure maintenance of substrate ATP levels within the myofibril. Muscles work less well in the absence of PCr [47–51]; further, the affinity of the myofibril for ATP is increased by the presence of PCr [48,51]. There is not yet any explanation of these observations. However, under the conditions of the experiments, and *in vivo*, ATP will be almost completely bound to Mg^{2+}, but the product of the ATPase reaction (ADP) will be only about 50% bound [41,42]. Hence if the energy were supplied by diffusion of ATP into the myofibrils, and diffusion of ADP back to the mitochondria, the $[Mg^{2+}]$ in the myofibrils would be locally raised, which would inhibit the Ca^{2+}-activation process. Similarly, if energy were to arrive by diffusion of phosphoenolpyruvate (PEP), Mg^{2+} would be similarly concentrated, since PEP, unlike pyruvate or phosphate, has an appreciable affinity for Mg^{2+} [27]. PCr has a very low affinity for Mg^{2+} (25–50 M^{-1}) [52], about the same as phosphate at pH 7.2, and supply of high-energy phosphate by its diffusion would not generate the local inhibitory rise in $[Mg^{2+}]$.

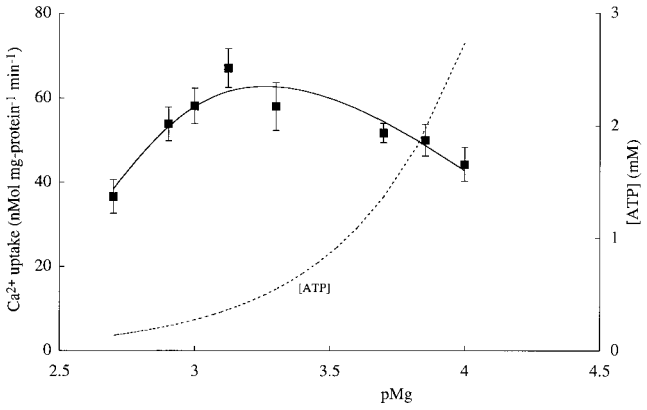


Figure 4 Effect of alterations in free Mg^{2+} concentrations on Ca^{2+} uptake into pig SR vesicles

The free [Mg^{2+}] was varied while the concentrations of MgATP (3.63 mM) and free Ca^{2+} (333 nM) were kept constant. Data are presented as means ± S.E.M. for six separate assays per point. The dashed line shows the change in free [ATP].

The sudden delayed onset of loss of cardiac contraction during ischaemia/anoxia

During the early 1990s the origin of the sudden but delayed loss of contractility of the heart during ischaemia was a subject of much discussion [3,53]. No single event was found to occur sufficiently markedly at the point of loss of contractility to account for its occurrence. It seemed likely that this loss of contractility was due to a cumulative effect of lowering of pH, increased phosphate and the loss of high-energy phosphate, although [ATP] was normally still more than sufficient for contraction [54], with an apparent dissociation constant of 13 μM. The best correlation was found with lowering of the [PCr]/[ATP] ratio [55–57]. The existence of the clearly demonstrated inhibitory competition of Mg^{2+} in the Ca^{2+} activation and the low [PCr]/[ATP] ratio suggests that under these conditions the high-energy phosphate is supplied by ATP diffusion with consequential local elevation of [Mg^{2+}]. This may therefore be the mechanism by which exhaustion of PCr leads to inhibition even before [ATP] is seriously depleted. There is also a strong possibility that the SR may also contribute to the loss of contractility at low [PCr]/[ATP] ratios via Mg^{2+} inhibition (see below).

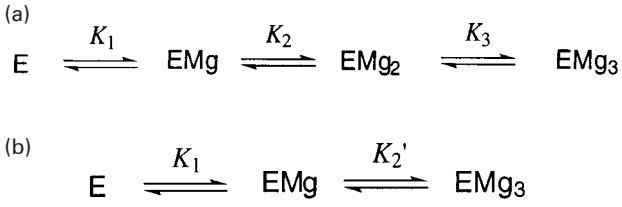
The substrate inhibition of myofibrillar ATPase observed at low [Ca^{2+}]

As reasoned in the previous section, an increase in MgATP and consequent decrease in the [PCr]/[ATP] ratio would lead to a changeover of energy supply to diffusion of MgATP. Substrate (MgATP) inhibition has been observed in skeletal muscle [58,59].

The cardiac SR and Mg^{2+}

Kinetic analysis of the uptake of Ca^{2+} by the SR

As [Mg^{2+}] is raised the rate of Ca^{2+} uptake by the SR first increases and then falls (Figure 4). As the experiment was performed at constant [MgATP], the activation phase (below 1 mM Mg^{2+}) is not derived from formation of MgATP. The inhibitory phase above 1 mM Mg^{2+} (left side of Figure 4) must be due to one or both of the variable concentrations, i.e. [Mg^{2+}] and $[ATP]_{free}$. The presence of PCr ensures there is no diffusion-generated gradient of [Mg^{2+}], so that the observed inhibition



Scheme 5 Activation and inhibition by Mg^{2+} of the SR Ca^{2+}-ATPase

(**a**) A minimum of three Mg^{2+} ions are involved. (**b**) The inhibition by Mg^{2+} is highly co-operative.

relates directly to the set concentration of Mg^{2+}. The SR and myofibril Ca-ATPases have similar kinetic properties, namely a greater dependence on [Ca^{2+}] than first order, and an unidentified, ATP-dependent, Ca^{2+}-binding site [60]. *In situ* there is also a similar negative response to the absence of PCr [61,62], for which the ATP regeneration system based on pyruvate kinase does not substitute [63]. These kinetic similarities, along with both the activation and inhibition by Mg^{2+}, led to the consideration of a scheme in which the apparent substrate for 'priming' of the Ca^{2+} pump is CaATP (Scheme 4, replacing M, for myofibril, with E for the SR Ca^{2+}-ATPase). A substrate Ca^{2+}-binding site for the SR ATPase has been suggested previously [60]. As with our treatment of the myofibril ATPase, it is a reasonable assumption that the activation and inhibition by Mg^{2+} arise from separate processes. We have therefore constructed a kinetic equation derived from first-order activation by Mg^{2+}. The fall in activity with an increase of [Mg^{2+}] was faster than could be explained by binding a single Mg^{2+} ion, so two further ions could be bound (see Scheme 5a).

Thus of the four species E, EMg, EMg_2 and EMg_3, it was assumed that only EMg was active, leading to the rate equation:

$$v = V_{max} \cdot K_1 \cdot [Mg^{2+}]/(1 + K_1 \cdot [Mg^{2+}] + K_1 \cdot K_2 \cdot [Mg^{2+}]^2 + K_1 \cdot K_2 \cdot K_3 \cdot [Mg^{2+}]^3) \quad (2)$$

where K_1, K_2 and K_3 are the (molecular) association constants for the first, second and third ions to bind. Since they are molecular constants, the singly ligated species EMg may have only some of its molecules with the Mg^{2+} bound in the activating site.

The experimental Ca^{2+} uptake values are shown in Figure 4 along with the theoretical curve obtained on fitting the above equation to the data. The values found for the variables used in the fitting are: $V_{max} = 75$ nmol·(mg of protein)$^{-1}$·min^{-1}, $V_{min} = 0$, $\log K_1 = 4.11$, $\log K_2 \cdot K_3 = 5.37$, $\log K_2 < 1$ and $\log K_3 \geqslant 5$. This solution indicates that there is either extreme co-operativity between the two inhibitory Mg^{2+} sites or that there are further inhibitory sites for Mg^{2+}. For the two-site model of inhibition the concentration of species with two Mg^{2+} ions bound is negligible and hence Scheme 5(a) may be simplified to Scheme 5(b). This gives the rate equation:

$$v = V_{max} \cdot K_1 \cdot [Mg^{2+}]/(1 + K_1 \cdot [Mg^{2+}] + K_1 \cdot K_2' \cdot [Mg^{2+}]^3) \quad (3)$$

where $K_2' = K_2 \cdot K_3 = 10^{5.37}$ M^{-2}, a second-order (molecular) association constant.

Alternatively, inhibition by Mg^{2+} is equivalent to activation by free ATP. For this study the [ATP] can be equated to 2.75×10^{-7} M^2/[Mg^{2+}]. Half inhibition occurs at pMg = 2.68, which is equivalent to half activation at $pATP_{free} = 3.87$. It is interesting to note that this affinity for free ATP, although clearly composite as shown by the co-operativity, is close to that of ATP for Ca^{2+}, and consistent with a kinetic scheme for activation (or priming)

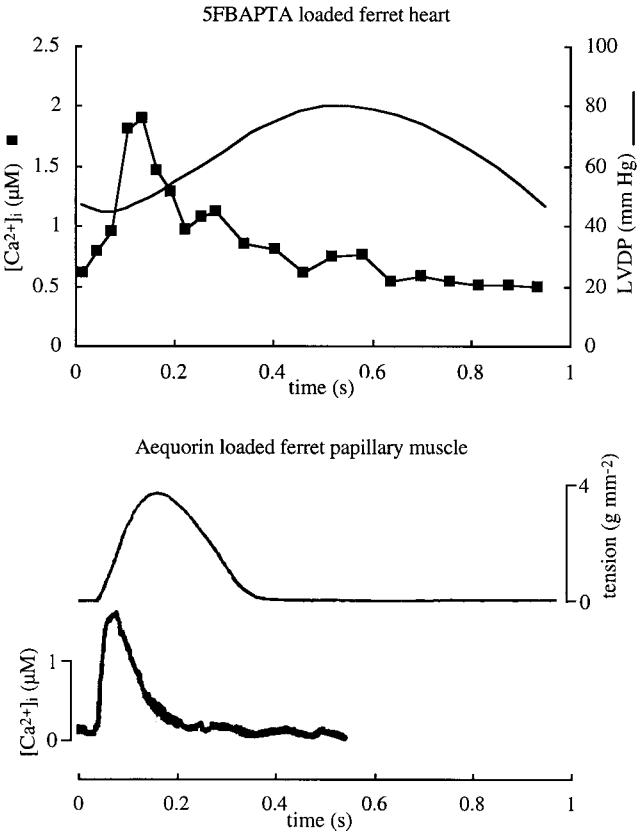


Figure 5 Comparison of the calcium and pressure transients in the presence and absence of exogenous cytoplasmic Ca^{2+} buffering

Upper panel: isolated ferret heart perfused with 5FBAPTA at 30 °C, with data from [69]. Lower panel: isolated ferret papillary muscle loaded with aequorin at 30 °C, with data taken from [70]. 5FBAPTA causes significant attenuation of both the Ca^{2+} transient and pressure transient, whereas aequorin causes minimum buffering of the Ca^{2+} transient and therefore minimum perturbation to the pressure transient. In both cases, however, LVDP appears to reach a peak as the Ca^{2+} transient returns to baseline.

where the equilibrium formation of CaATP is the dominant factor. This is corroborated by the fact that $[Ca^{2+}]$ is about 0.4 mM for half activation of the initial phosphoenzyme production when [ATP] is very low; as may be calculated from Figure 2(a) in [64].

The final step of the ATPase reaction is the liberation of P_i. The ATPase reaction is inhibited by P_i (product inhibition) and this is reflected in inhibition by vanadate, which acts as an analogue of P_i. The vanadate effect is modulated by Mg^{2+} and hence it is not unreasonable to assume that Mg^{2+} affects the competitive binding of the vanadate ion at the active site. This would suggest that Mg^{2+} interacts with P_i bound to the active site and promotes its release. For this model one may write a Michaelis–Menten equation of the form:

$$\text{Rate of } P_i \textit{ production}, v = [\text{E-P}_i]\cdot K_b\cdot[Mg^{2+}]/(1+K_b\cdot[Mg^{2+}]) \quad (4)$$

where E-P_i is the penultimate species before phosphate release. In the present case we may also write a Hill equation for the concentration of E-P_i in terms of the inhibitory action of Mg^{2+}. The above equation then becomes;

$$v = V_{max}\cdot[E]\cdot K_b\cdot[Mg]/(1+K_b\cdot[Mg^{2+}])(1+K_c\cdot[Mg^{2+}]^n) \quad (5)$$

If n is set to 2 during fitting, this equation reduces to that derived above for the three-step model. When n is allowed to vary, the best fit to the data yields values of $V_{max} = 68.8$ nmol·(mg protein)$^{-1}$·min^{-1}, $\log K_b = 4.24$, $\log K_c = 3.88$ (ATP activation), $\log K_i = 2.68$ (Mg^{2+} inhibition) and $n = 3.1$.

Greater co-operativity of inhibition would be required if the activation by Mg^{2+} were assumed greater than first order. The results of fitting these modified equations are essentially indistinguishable from that shown in Figure 4.

Further similarities of the kinetics of SR and myofibril ATPases

The equation derived for the Mg^{2+} activation of the SR ATPase (eqn 4) is of the same form as that which fits the V_{max} dependence on $[Mg^{2+}]$ at saturating Ca^{2+} that emerged from the myofibrillar ATPase data processing above (eqn 1). The myofibrillar ATPase is another enzyme where vanadate can mimic the leaving phosphate group and this has been much used in the study of intermediates. Hence, we may also infer the involvement of activating Mg^{2+} at the phosphate-release stage and the K_m values for the two enzymes are very similar. The full affinity of the cation–nucleotide–enzyme complexes for the activating Mg^{2+} probably results from the increased negative charge arising with scission of the nucleotide β–γ bond. This is consistent with the binding of Mg^{2+} as the final reaction step leading to the release of P_i.

The co-operativity of the Mg^{2+} inhibition (ATP activation) of the SR Ca^{2+}-transporting ATPase mirrors the similar Mg^{2+}/ATP kinetics already implicated above in the activation of the myofibrils. In addition to the required CaATP formation (first-order inhibition with respect to Mg^{2+}), appreciable contributions of either the inactive (unprimed) ECaMgATP or EMgATP (both first order with respect to Mg^{2+}) would increase the possible Hill coefficient for Mg^{2+} inhibition above unity. There is also evidence for a direct inhibitory binding of Mg^{2+} at the identified enzyme Ca^{2+}-transporting site [65], which would lead to an even higher Hill coefficient for Mg^{2+} inhibition.

Myofibril activation and the Mg^{2+} inhibition of the SR Ca^{2+}-ATPase

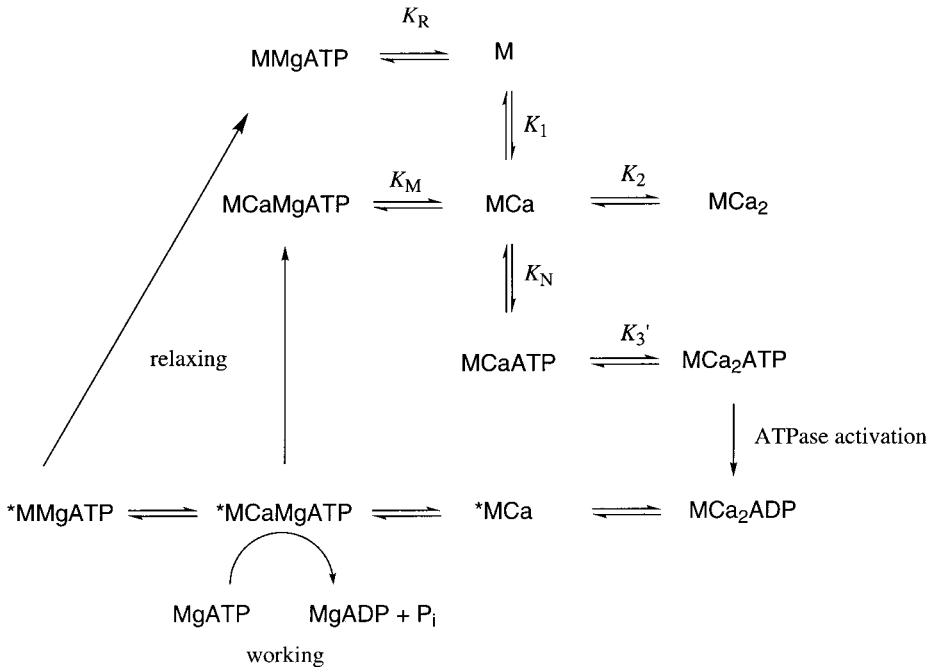
Raised Mg^{2+} concentrations in the cell might not only have direct effects on the myofibril, but could conceivably block many cellular transport systems, especially those relating to Ca^{2+} [9,66]. The SR could affect the contractile process either directly through the release or uptake of Ca^{2+} or by other effects, as follows.

Secondary effects of SR function on contraction

The SR Ca^{2+}-transporting ATPase lies within the same diffusion-limited region as the myofibrils and has the same requirement as the contractile apparatus; i.e. it must not pump Mg^{2+} into the region by acquiring its energy supply by diffusion of MgATP. Indeed it has many of the properties of the myofibril ATPase, having a positively co-operative activation by Ca^{2+} that is ATP-dependent [67], a requirement for PCr and sensitivity to inhibition by Mg^{2+}.

Direct effects of Mg^{2+} on SR Ca^{2+} loading and resulting release

The kinetic parameters of the cardiac SR Ca^{2+}-ATPase predict that a local rise in $[Mg^{2+}]$, which would result from a low [PCr]/[ATP] ratio, e.g. during ischaemia, could also harmfully lower the rate of Ca^{2+} loading of the SR. Under normal physiological conditions there is more than adequate time for the cardiac SR to be near-maximally loaded with Ca^{2+}, and this ensures sufficient Ca^{2+} release for maximal LVDP. However,



Scheme 6 The complete kinetic scheme for myofibril Ca^{2+}-ATPase activation

*M represents the myosin when a cross-link to actin has been established. The species *MCa has a cross-bridge to actin and by implication contains CaADP, which is replaced by MgADP during the first working cycle. Since the troponin dissociates on forming the cross-bridge, the Ca shown in each of the species containing *M must be bound to the free myosin head group.

given sufficient suppression of uptake rate by raised $[Mg^{2+}]$, a point would be reached when the loading of Ca^{2+}, and consequentially its later release, would no longer be maximal. This would augment the direct suppression of the contractile ATPase by raised $[Mg^{2+}]$, and lead to an enhanced reduction in LVDP.

Recruitment and energy consumption

The separation of enzyme initiation and functional activity

In addition to the considerations above, there are further mechanisms that may generate a response to changes in $[Mg^{2+}]$. From the known affinities of other sites for bivalent cations, e.g. the type I Ca^{2+}/Mg^{2+} sites on troponin, Mg^{2+} effects are expected to appear under appropriate conditions. Alternatively it is possible that, under conditions of fixed $[ATP]_{free}$, Mg^{2+} activation may arise from the subsequent use of MgATP as substrate when work is performed by the enzymes after the initiation has occurred.

In a series of papers on muscle, He and others [37,68] introduced an extremely rapid continuous assay of phosphate generation, based on a phosphate-binding protein that carried a fluorescent label. This assay, along with essentially instantaneous initiation of the activity by laser-induced release of either ATP or Ca^{2+} from caged complexes, produced an unexpected result. In all the muscle types studied (including cardiac [68a]), the ATPase activity showed two distinct phases, a short, fast initial phosphate release followed by a slower prolonged rate. In some instances the switch of rates occurred sharply after an estimated release of two phosphates per myosin. The slowing of ATPase rate was not due to build up of products (P_i, Mg^{2+} or ADP). Our proposal of separate apparent substrates for initiation and working ATPase is consistent with these observations.

The temporal relationship of the cardiac $[Ca^{2+}]$ and contraction transients

In our own work the clearest indication of the complexity of Ca^{2+} activation, as described above, is found when comparing under different conditions the relationship of the time course of the Ca^{2+} transient with that of the associated contraction. Figure 5 shows this comparison in the presence and absence of added Ca^{2+} buffering, the heavily buffered heart, using 5FBAPTA, taken from Harding et al. [69] (upper panel), and the very weakly buffered aequorin-loaded heart muscle, taken from Yue et al. [70] (lower panel), both plotted on the same time scale. We do not show the data acquired before the loading of 5FBAPTA, when the contraction resembles that for aequorin with a delay of only a few milliseconds, due to the mechanics of the measurement procedure. In each case the maximum contractile strength occurs after the initial fall from peak systolic $[Ca^{2+}]_i$, both when the $[Ca^{2+}]_i$ is unbuffered and the transients are brief, and when it is heavily buffered and the transients are much longer. The peak of contraction occurs when the $[Ca^{2+}]_i$ has nearly reached diastolic level, making the delay in peak contraction appear to depend on the speed of decline of the $[Ca^{2+}]_i$. This is consistent with a requirement for switching the substrate from CaATP to MgATP after activation. The muscle fibre and the balloon-catheterized heart are probably not truly isometric and some physical movement against developing force, via cross-bridge movement along the actin filaments, is required before the peak of force is observed.

Scheme 6 shows the basic equilibria required by the kinetics of the system indicated in Figure 5. The species MCaMgATP, previously considered to be the active form of the ATPase, may form a cross-bridge but very much more slowly than MCa_2ATP, which does so in the reactions represented on the bottom right of Scheme 6. This makes the relaxation (upwards arrows further

left) essentially irreversible. The species *MCa has a cross-bridge to actin and by implication contains CaADP, which is replaced by MgADP during the first working cycle. This is effectively a Ca^{2+}-dissociation step after cross-bridge formation (M to *M), which is consistent with the relative time courses of the transients in highly Ca^{2+}-buffered hearts. Since the troponin dissociates on forming the cross-bridge, the Ca shown in each of the species containing *M must be bound to the free myosin head group and the working cycle thus switches between the two myosin heads, implying alternate bridges to the actin filament. In the absence of Mg^{2+} the substrate CaATP may be consumed, but at a lower rate, possibly reflecting a Mg^{2+} requirement for the P_i dissociation step, or because of the relative rates of the cross-bridge dissociation when it contains Mg^{2+} or Ca^{2+}. The kinetic data analysed by Petushkova [71] can also be explained by MgATP/CaATP competition in the cycling. Hoskins and colleagues [72] recently showed, using a photo-activated Ca^{2+} chelator, that the relaxation step, *MMgATP to MMgATP in the model in Scheme 6., is much faster than the previously considered cross-bridge cycling rate, *MCaMgATP to MCaMgATP in this model.

The kinetic scheme for SR Ca^{2+}-ATPase activation

A proposal for the SR Ca^{2+}-transporting ATPase is also shown in Scheme 6 (E replaces M). As with the myofibril, CaATP is the substrate for activation, but MgATP is needed for continued pumping. This means that the pumping of Ca^{2+} back into the SR does not start until other processes, such as Na^+/Ca^{2+} exchange at the cell membrane, have lowered the $[Ca^{2+}]_i$. Only then does efficient pumping start, so that the transient rise of $[Ca^{2+}]$ is not diminished by premature SR pumping. This is consistent with the unaltered total Ca^{2+} release from the SR, over an extended time course, when the exogenous buffering is added [10].

Numerically the SR ATPase follows the kinetics of the myofibril. However, the structure in a membrane environment and the function are clearly very different. The basis for the similar kinetics is unknown. Since the K_m for ATP does not diminish indefinitely as $[Ca^{2+}]$ is raised, a species (ECa_2) must exist, as well as ECa_2ATP (Scheme 6). Indeed, evidence exists that the functional enzyme unit is dimeric [73–75], the monomer and higher oligomers being inactive [76]. The dimers can show non-co-operative or positively co-operative Ca^{2+} binding [67], presumably related to the absence or presence of ATP. During reloading in diastole the SR ATPase is fully active. The comparison with the myofibril ATPase would suggest that an unprimed state of the SR Ca^{2+}-transporting ATPase, requiring similar co-operative Ca^{2+} activation and inhibited by Mg^{2+}, is established at end diastole or during the release phase of the cycle.

Conclusions

Our considerations imply that two troponin molecules, and hence two myosin molecules, take part in the initial cross-bridge formation. This initiating condition is manifestly different from the situation when one head is already attached to actin and the associated troponin has dissociated. When one cross-bridge is already present, the activation by binding of Ca^{2+} to the remaining troponin on the free myosin head, and subsequent cross-bridge formation by MgATP in the contraction step, must allow the first cross-bridge to dissociate. This is clearly a requirement for the dissociation of ADP and the rebinding of troponin and Ca^{2+} before the system is able to cycle. This alternating model is an attractive idea, because the functional complex has two myosin heads. Indeed, freeze-fracture techniques indicate that the contracting myofibril has only one of the heads firmly attached to actin at any time, whereas the system in rigor has both heads attached [11,12]. Thus the rigor state is formed by the further action of CaATP on the species *MCa in Scheme 6.

The continued function of the heart is central to survival of the organism and it is self-reliant for the supply of the raw materials required to energize this function. The contractile function (apparent dissociation constant for ATP = 13 μM) is far the greater part of the ATP-consuming ability of the myocyte. The cells also need energy in the form of ATP for many vital processes, most notably phosphorylation to provide further ATP production; the ATP-dissociation constants (K_m values) are for hexokinase 200 μM and for phosphofructokinase 75 μM. Systems will therefore exist in the myocyte to protect these other vital functions from a catastrophic, possibly lethal, contraction-driven decline in the [ATP]. Such a system would require a controlled reduction in the response to functional demands on energy (ATP) when they are high relative to the supply of raw material (e.g. O_2). The inhibition of the Ca^{2+} activation of the major muscle ATPases observed at low [PCr]/[ATP] ratios affords this type of protection.

The SR Ca^{2+}-uptake experiments were carried out in the laboratory of Roland Vetter, Department of Molecular Cardiology, Max Delbrück Centre for Molecular Medicine, Berlin-Buch, Germany. J.I.V. is a British Heart Foundation Basic Sciences Lecturer.

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