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Parvalbumins and muscle relaxation: a computer simulation study

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Summary

The distribution of Ca^{2+} and Mg^{2+} among the 'regulatory' cation binding sites of troponin (T-sites) and the strong, Ca^{2+}–Mg^{2+} binding sites of troponin and parvalbumins (P-sites) in the sarcoplasm of a muscle was calculated. At rest, 60% of the T-sites were metal free, while 92% of the P-sites were loaded with Mg^{2+}.

In response to a Ca^{2+} pulse, troponin-calcium (T–Ca) complexes were rapidly formed, while the binding of Ca^{2+} to P-sites was limited by the slow rate of dissociation of the parvalbumin–magnesium (P–Mg) complexes. Muscle activation was not prevented by a high content of parvalbumins.

Parvalbumin and the sarcoplasmic reticulum (SR) pump were complementary relaxing factors that removed Ca^{2+} from the cytosol and from the T-sites. Parvalbumins dominated the first part of relaxation, while the action of the SR was essential to ensure the return to a very low level of free Ca^{2+} ion and of T–Ca. After relaxation, a large fraction of the Ca^{2+} pulse was still bound to parvalbumins and returned slowly to the SR during the recovery.

When the SR activity was reduced, the presence of parvalbumins preserved a fast rate of relaxation, at least for a few contractions. This may have a high adaptive value in cold-blooded animals.

Introduction

Parvalbumin has been studied extensively as a prototype for homologous calcium-modulated proteins, such as calmodulin and troponin C. Its own function remains unknown, even though many functions have been suggested over the past decade. The initial sources of parvalbumin were frog and carp. Hamoir (1968) suggested that 'they seem to be necessary as long as semipermeable membranes exist'. Only subsequently

Ed. notation: Free calcium ion (free Ca^{2+}); bound calcium ion (bound Ca^{2+}).

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was parvalbumin isolated from terrestrial mammals in whose muscles it exists in very low concentration.

Parvalbumin is not an enzyme: its stucture has no 'pits' or 'crevasses' characteristic of all enzyme structures determined to date; and Pechère & Focant (1965) found no evidence of enzymatic activity when parvalbumin was tested for a variety of possible reactions associated with glycolysis.

Following the realization that parvalbumin binds calcium, Pechère *et al.* (1971) suggested that 'it might have biochemical effects similar to those of troponin and also that chemical isologies might be detected . . .'. Subsequently, it has been found that the fast white muscle of fish, where parvalbumin occurs in greatest abundance, also contains troponin. It does not replace troponin or myosin light chains in myosin ATPase assay systems *in vitro* (Demaille *et al.*, 1974b). Blum *et al.* (1974) reported a phosphorylated form of parvalbumin which might provide a clue to its function but this appears to have been an artifact (Demaille *et al.*, 1975); any phosphate acceptor protein in the myogen fraction is not parvalbumin. Potter *et al.* (1977) found a calcium-dependent activation of brain cyclic nucleotide phosphodiesterase by parvalbumin at 2000 times the molar concentration of calmodulin required for activation. Considering the high levels of parvalbumin present in fish muscle, they suggested that it 'may regulate skeletal muscle phosphodiesterase'. Pechère *et al.* (1975) suggested that parvalbumin might interact with a membrane ATPase. Using indirect immunofluorescence, Heizmann *et al.* (1977) saw 'regular cross-striation patterns within the I-band of isolated myofibrils' of chicken and suggested that 'a parvalbumin-like protein may interact with one or more of the protein components of the thin filament'. The three preceding suggestions require a direct interaction of parvalbumin with another protein. Such an interaction has not been demonstrated.

Hitchcock & Kendrick-Jones (1975) found that neither parvalbumin nor myosin light chains can substitute for troponin C in functional assays or in troponin I and tropomyosin binding studies. They then suggested that parvalbumin serves as a soluble store of bound calcium in the sarcoplasm. Pechère *et al.* (1975) elaborated upon this theme and argued that parvalbumin functions as an H^+-dependent Ca^{2+} buffer. That is, parvalbumin would not bind unless the pH decreased as a result of ATP hydrolysis. Pechère *et al.* (1977) subsequently withdrew this interpretation when no pH dependence for calcium binding was observed. Briggs (1975) suggested that parvalbumin acted as a soluble relaxing factor in muscle. Gerday & Gillis (1976) and Blum *et al.* (1977) determined that fragmented sarcoplasmic reticulum (SR) could deplete parvalbumin of calcium, and that parvalbumin in turn could deplete myofibrils of calcium. These observations are consistent with a 'trap or carrier' idea of Demaille *et al.* (1974a). The affinity of parvalbumin for calcium is greater than that of troponin but less than that of the SR pump. However, the rate of calcium binding by troponin may exceed that of parvalbumin. Hence, upon release from the SR, calcium would first bind to troponin and activate myosin ATPase. It would then be complexed quickly by parvalbumin and 'stored' during muscle relaxation, while the SR pump subsequently removes

the calcium (Gillis & Gerday, 1977). The binding and rate constants published during the last few years permit us to evaluate quantitatively this 'relaxation' model of parvalbumin function.

During the course of this work, Robertson *et al.* (1981) published a computer simulation study on the time course of Ca^{2+} exchange with troponin and parvalbumin in response to a free Ca^{2+} transient. The present results fully confirm their conclusion concerning the rate of Ca^{2+} binding to the Ca^{2+} specific sites (T-sites) using similar concentrations and kinetic parameters but do not support the conclusion that parvalbumin is unable to act as a relaxing factor *in vivo*. As shown in this paper, at the concentrations found in amphibian and fish fast muscles, parvalbumin can contribute to relaxation by facilitating Ca^{2+} dissociation from troponin. This effect may be particularly important in delaying the onset of tetanus during a 'burst' of electrical activity. A preliminary report of some of this work has already appeared (Gillis, 1980).

The model and its components

The model consists of three components, which are described in detail below: troponin, parvalbumin and SR. Firstly, the situation of the resting muscle will be evaluated by calculating the equilibrium distribution of the Ca^{2+} or Mg^{2+} complexes and the apo-forms of troponin and parvalbumin at the concentrations of free Ca^{2+} and Mg^{2+} prevailing in the resting state. Secondly, the perturbation of this equilibrium produced by a 'pulse' of calcium into the system will be studied as a model for muscle activation. As a result of this Ca^{2+} pulse, there will be a temporal redistribution of calcium and magnesium among the various binding sites of troponin and parvalbumin together with the eventual sequestration of this calcium by the SR. Finally, the simulations will be extended to the case of repetitive stimulations. These studies will thus provide an evaluation of the relative importance of parvalbumin and the SR in removing Ca^{2+} from troponin, and hence in producing relaxation.

Troponin

The troponin content of vertebrate skeletal muscle, is about 70 μM (Ebashi *et al.*, 1969) and through its TN-C subunit troponin confers calcium-sensitivity to the actin–myosin interaction. TN-C contains four divalent metal binding sites which can be divided into two classes. The first one, often called 'Ca-Mg', has a very high affinity for Ca^{2+} ($K = 10^{-8}$M) and a 10^4 times lower affinity for Mg^{2+} (Potter & Gergely, 1975). The latter affinity is, however, high enough to give high concentration of the TN-C–Mg complex at the free Mg^{2+} concentration ($10^{-2.5}$M) found in resting muscle (Cohen & Tyler-Burt, 1977). These sites apparently have no regulatory role, and are designated 'P-sites', because of the similarity in binding affinities and rate constants to the divalent cation binding sites present in parvalbumin.

The second class of divalent metal ion binding sites, 'Ca^{2+}-specific' or 'regulatory', have a lower affinity for Ca^{2+} ($K_d = 10^{-6.5}$M when TN-C is complexed with TN-I and TN-T to form whole troponin) and again a 10^4 times lower affinity for Mg^{2+} (Johnson *et*

al., 1979). It is often assumed, for instance by Robertson *et al.* (1981), that this low affinity for magnesium can be neglected and the sites considered as calcium specific. As will be shown, this approximation is only partially justified. Functionally, these sites are essential in muscle regulation (Potter & Gergely, 1975) and have been designated 'T-sites'.

Parvalbumin

Parvalbumins are a class of soluble calcium-binding proteins found principally in striated muscle. In contrast to troponin, their concentrations vary from muscle to muscle and species to species. Fast skeletal muscles of fishes and amphibians have the highest content (ranging from 0.35 to 1.5 mM) (Table 1), while in avian and mammalian muscles, the concentration is 10–20 times lower. Parvalbumin has two metal binding sites, which are of the high affinity, Ca–Mg type (Potter *et al.*, 1978) already described for TN-C and called here 'P-sites'. Here, all 'P-sites' are treated as a single class whether they belong to troponin or to parvalbumin.

It is assumed that the binding of Ca^{2+} or Mg^{2+} to either class of site is non-cooperative, and shows straight competition between Ca^{2+} and Mg^{2+} (Cox *et al.*, 1977). All sites were treated as individual molecules, with no diffusion restriction.

Sarcoplasmic reticulum

(1) The amount of Ca^{2+} liberated by the SR in response to stimulation must be at least 140 μmol Ca^{2+} l^{-1}, if the regulatory sites of troponin have to be saturated. From the

Table 1. Constants and parameters used in the calculations (temperature 20° C).

	Ca^{2+}			Mg^{2+}		
	K_d (M)	k_{off} (s^{-1})	k_{on} (M^{-1} s^{-1})	K_d (M)	k_{off} (s^{-1})	k_{on} (M^{-1} s^{-1})
T-sites	$10^{-6.5}$	$10^{1.5}$ [a]	$10^{8.0}$ [a,b]	$10^{-2.3}$	$10^{2.3}$ [b]	$10^{4.6}$ [b]
P-sites	$10^{-8.0}$	$10^{0.0}$ [a]	$10^{8.0}$ [a,b]	$10^{-4.1}$	$10^{0.5}$ [c]	$10^{4.6}$ [c]

Concentrations (10^{-3}M): T-sites from troponin 0.14[d,e]
P-sites from troponin 0.14[d]
P-sites from parvalbumin 0.70 (frog, carp)[f] 1.20 (hake, pike)[g]; 3.00 (swimbladder muscle)[h]

Sarcoplasmic reticulum: $K_m = 10^{-7}$ M Ca^{2+} [i]
V_{max} (25° C): 70 μmol Ca^{2+}/g SR protein/s[i], $Q_{10} = 3$[j]
Content: 10g SR protein/l fresh muscle[k]

Free ion concentration at rest: $Ca^{2+} = 10^{-7.7}$ M[l]; $Mg^{2+} = 10^{-2.5}$ M[m]
Calcium release per stimulus: 200 μmol l^{-1} [n]. Repetitive stimulations: 2 s^{-1}

[a]Johnson *et al.* (1979); [b]Diebler *et al.* (1969), see text; [c]Potter *et al.* (1980); [d]Potter & Gergely (1975); [e]Ebashi *et al.* (1969); [f]Gosselin-Rey & Gerday (1977), Hamoir (1968); [g]Baron *et al.* (1975), Bhushana Rao & Gerday (1973); [h]Hamoir *et al.* (1980); [i]Inesi & Scarpa (1972); [j]Weber *et al.* (1966); [k]Peachey (1965); [l]see text and Ashley & Campbell (1979); [m]Cohen & Tyler-Burt (1977); [n]Endo (1977), Ashley *et al.* (1965).

relation between tension and Ca^{2+} concentration, Endo (1977) estimated that 210 μmol Ca^{2+} l^{-1} are needed. Using Ca^{2+} buffers injected intracellularly, Ashley *et al.* (1965) concluded that 200–500 μmol Ca^{2+} l^{-1} are liberated and a value of 200 μM was used by Ashley *et al.* (1974) in their subsequent kinetic analysis. In the present paper, a value of 200 μmol Ca^{2+} l^{-1} has been used as the amount liberated by a single action potential.

The kinetics of this liberation is unknown. In a preliminary report (Gillis, 1980), it was simulated by an instantaneous (step function) injection of Ca^{2+}. The Ca^{2+} pulse is now modelled in a more realistic way, using Equation 1 which allows the expansion of the Ca^{2+} pulse in time:

$$(Ca^{2+} \text{ entry})_t = 200\ \mu\text{mol } Ca^{2+}\ l^{-1}\ (1 - Ae^{-t/\alpha} + Be^{-t/\beta}). \quad (1)$$

The numerical values of the parameters have been adjusted until the results of the simulations showed that (a) the free Ca^{2+} concentration remains below 10 μM Ca^{2+}, in agreement with the results from both aequorin-injected frog fibres (Blinks *et al.*, 1977) and crustacean muscle (Ashley *et al.*, 1974); (b) the pulse allows at least 90% saturation of the T-sites with Ca^{2+}; and (c) the highest T–Ca concentration is obtained within 20 ms. In simulations meant to mimic changes in frog muscle, these conditions are fulfilled (see Results and Fig. 2, curves b) for the following values: time constants – $\alpha = 6.0$ ms; $\beta = 0.8$ ms; dimensionless constants – $A = 1.1525$; $B = 0.1525$. These were adopted for all simulations reported here.

(2) The Ca^{2+} uptake by the SR obeys typical Michaelis–Menten kinetics, with a K_m around 10^{-7}M Ca^{2+} (Inesi & Scarpa, 1972). The V_{max} for this process can be taken as the initial rate of Ca^{2+} uptake determined *in vitro* by Inesi & Scarpa (1972) employing rapid mixing techniques, Ca^{2+} indicators and a saturating Ca^{2+} concentration of 0.1 mM. In these conditions, a rate of 70 nmol Ca^{2+}/mg SR protein/s has been obtained for mammalian SR, at 25° C. The temperature coefficient (Q_{10}) for Ca^{2+} uptake and the associated ATP hydrolysis is close to 3.0 (Weber *et al.*, 1966; Inesi & Watanabe, 1967).

The amount of the SR in fast skeletal muscles estimated from serial electron micrographs reaches 10 mg g^{-1} wet muscle weight (Peachey, 1965). Hence a value of 700 μmol Ca^{2+} l^{-1} has been adopted as the V_{max} of the uptake step. From experiments on skinned frog fibres, Endo (1977) gives an uptake rate of 230 μmol Ca^{2+} l^{-1} s^{-1}, at 15° C, a figure in good agreement with the one used here, after correction for the temperature difference.

Resting conditions

Various workers have measured the concentration of free Ca^{2+} in muscle cells using aequorin injection, calcium-selective microelectrodes, and through an evaluation of the K_m (Ca^{2+}) of isolated SR vesicles (see Ashley & Campbell, 1979). Most of the reported values range from 1.0×10^{-8} to 3.0×10^{-7}M. We use a value of 2.0×10^{-8}M.

The concentration of the free Mg^{2+} is above 10^{-3}M and is probably close to that of the extracellular fluid. A value of 3 mM was deduced from the n.m.r. study of Cohen & Tyler-Burt (1977) and has been used here. Much of the magnesium of the cell is complexed with organic ions, mainly ATP and myosin. However, the dissociation of

Mg–myosin is very slow ($k_{off} = 0.06\ s^{-1}$; Potter *et al.*, 1980) and a change of the Mg–ATP concentration during a twitch has not been detected *in vivo* presumably because of rapid ATP regeneration from phosphocreatine. Therefore, it has been assumed here that the concentrations of these complexes remain constant.

Binding of divalent ions to the T- and P-sites

The numerical values used in this paper are summarized in Table 1. When an 'off' or 'on' rate constant had not been determined experimentally, it was deduced from the relation $K_d = k_{off}/k_{on}$. Two points have to be emphasized concerning the T-sites. Firstly, the affinity constant for Ca^{2+} is increased ten times when TN-C is combined with the other subunits of troponin (Johnson *et al.*, 1979). The on rate cannot increase as it is limited by the dehydration rate of the aquo-Ca^{2+} ion $[Ca(H_2O)_{6-8}]^{2+}$, and as k_{off} for water is $10^{8.0}\ s^{-1}$ (Diebler *et al.*, 1979), thus the off rate measured for TN-C must be reduced by ten times for whole troponin (assuming that Ca^{2+} binding occurs in a single step reaction). Secondly, the rate constants for T–Mg have not been determined and only the K_d is known. It has been argued that the dehydration rate of the aquo-Mg^{2+} ions ($10^{4.6}\ s^{-1}$; Diebler *et al.*, 1969) places an upper limit to the on rate of binding of Mg^{2+} to a protein site and this value has been taken as the on rate constant for the T–Mg formation.

Repetitive stimulations

The effect of a series of Ca^{2+} pulses, at the frequency of $2\ s^{-1}$ was examined. In frog muscles, at room temperature, this stimulation frequency gives well-separated twitches. In the absence of data concerning the absolute amount of Ca^{2+} liberated *in vivo* from pulse to pulse, the Ca^{2+} pulse was kept constant throughout. This certainly represents an extreme situation for *in vivo*: (1) the Ca^{2+} store of the SR is limited [the rapidly exchangeable Ca^{2+} is estimated at about $700\ \mu mol\ l^{-1}$ (Endo, 1977)]; and (2), the light emission signal from aequorin-injected fibres steadily diminishes in a series of twitches (Blinks *et al.*, 1977).

Numerical analysis

The resting state

The distribution of calcium and of magnesium in the resting state is itself of importance as it is the starting point for calculation of the transient variations. The distribution is described by four conservation and four equilibrium equations. There are eight unknowns and eight constants as listed in Table 1.

$$[\Sigma P] = [P] + [PCa] + [PMg] \tag{2}$$

$$[\Sigma T] = [T] + [TCa] + [TMg] \tag{3}$$

$$[Ca] = [PCa] + [TCa] + [Ca^{2+}] \tag{4}$$

$$[Mg] = [PMg] + [TMg] + [Mg^{2+}] \quad (5)$$

$$K_d(T/Ca) = [T] \cdot [Ca^{2+}] \cdot [TCa]^{-1} \quad (6)$$

$$K_d(T/Mg) = [T] \cdot [Mg^{2+}] \cdot [TMg]^{-1} \quad (7)$$

$$K_d(P/Ca) = [P] \cdot [Ca^{2+}] \cdot [PCa]^{-1} \quad (8)$$

$$K_d(P/Mg) = [P] \cdot [Mg^{2+}] \cdot [PMg]^{-1} \quad (9)$$

The K_ds are the dissociation constants relevant to each site–metal reaction [for example, (T/Ca), T-sites and calcium], while [T] and [P] denote the apo-forms of the sites.

At equilibrium, the relative concentrations of the calcium and magnesium complexes are calculated by the following equations

$$\frac{[BCa]}{[\Sigma B]} = \frac{x}{1 + x + y} \quad (10)$$

$$\frac{[BMg]}{[\Sigma B]} = \frac{y}{1 + x + y} \quad (11)$$

where B is the binding site in consideration (T or P).

$$x = \frac{[Ca^{2+}]}{K_d(B/Ca)} \quad (12)$$

and

$$y = \frac{[Mg^{2+}]}{K_d(B/Mg)} \quad (13)$$

The excited state

Following the 'injection' of calcium, defined by Equation 1, the concentration of the ions bound to the T- and P-sites are described by four differential equations. A fifth equation describes the sequestration of calcium by the SR.

$$d[TCa]/dt = -k_{off}(T/Ca) \cdot [TCa] + k_{on}(T/Ca) \cdot [T] \cdot [Ca^{2+}] \quad (14)$$

$$d[TMg]/dt = -k_{off}(T/Mg) \cdot [TMg] + k_{on}(T/Mg) \cdot [T] \cdot [Mg^{2+}] \quad (15)$$

$$d[PCa]/dt = -k_{off}(P/Ca) \cdot [PCa] + k_{on}(P/Ca) \cdot [P] \cdot [Ca^{2+}] \quad (16)$$

$$d[PMg]/dt = -k_{off}(P/Mg) \cdot [PMg] + k_{on}(P/Mg) \cdot [P] \cdot [Mg^{2+}] \quad (17)$$

$$d[Ca]/dt = -V_{max} \cdot [Ca^{2+}] \cdot (K_m + [Ca^{2+}])^{-1} \quad (18)$$

The kinetic equations are first-order, non-linear differential equations and are not directly integrable. In addition, the equations were determined to describe a system that is *stiff*, that is, one component changes on a time scale wholly different from at least one other component. Following the pulse of calcium, both apo-T-sites and apo-P-sites bind

calcium much faster than the SR can pump calcium or than magnesium can dissociate from magnesium-P-sites. For a complete discussion of stiff systems see Shampine & Gear (1979) and Ralston & Rabinowitz (1978).

The Charlottesville group have used Gear's method (Gear, 1971) to solve these stiff differential equations, while the more traditional fourth-order Runge–Kutta integration routine (McCraken & Dorn, 1966) has been used by the Brussels group. The results obtained were the same, provided that the integration step in the Runge–Kutta method was kept very small (4×10^{-5} s). However, in terms of computer time, Gear's method achieves a 90% saving relative to the traditional approach and is preferred for this type of problem.

Results

The resting state

The concentration of the calcium and magnesium complexes, as well as the apo-form of T-sites and P-sites are given in Table 2. They were calculated using the concentrations and constants given in Table 1. Four cases have been considered: (1) muscle without parvalbumin (P-sites from troponin only); (2) amphibian muscle; (3) fish muscle (fast, white); and (4) the muscle of the swimbladder of the fish, *Opsanus tau*, which has the highest parvalbumin content reported so far (Hamoir *et al.*, 1980).

Fifty-nine per cent of the T-sites are present in a metal-free or apo-form, and 37% form a complex with magnesium. This implies that full activation of the muscle (100% T–Ca) will be partly limited by the dissociation rate of the T–Mg complex.

Ninety-three per cent of the P-sites are occupied by magnesium, 4.7% are in the form of P–Ca and only 2.3% are metal-free. Although this concentration of apo-P-sites is very small compared with the total P-site content, it is not negligible when compared with the concentration of apo-T-sites. In amphibian muscle the ratio apo-T/apo-P is 4.2 but is reduced to 1.1 in the swimbladder of *Opsanus tau*. As the on rate constants for calcium binding to apo-T and to apo-P sites are the same, a direct competition between these two types of sites for the calcium pulse can be expected (see Fig. 3).

Table 2. Distribution of apo- and metal forms of T- and P-sites in resting conditions. All concentrations in μmol l^{-1}.

	T-sites (total)	*Apo-T*	*T–Ca*	*T–Mg*
All muscles	140	82.6	5.3	52.1
	P-sites (total)	*Apo-P*	*P–Ca*	*P–Mg*
No parvalbumin	140	3.3	6.5	130.2
Frog, carp white muscle	840	19	41	780
Hake, pike white muscle	1340	31	62	1247
Swimbladder muscle (toad fish)	3140	73	146	2921

Response to a single calcium pulse

In this section the concentration of the various forms of T-sites and P-sites in response to a single calcium pulse have been calculated, starting from the resting conditions in Table 2. For all simulations, the total concentration of the T-sites, and the size of the calcium pulse are kept constant. The concentration of P-sites and the V_{max} of the SR was varied in order to study the effect of these parameters on the contraction–relaxation cycle.

It is assumed that muscle activation is directly related to the concentration of the T–Ca complex; consequently; the disappearance of this complex will be called 'relaxation'.

Relaxing effect of P-sites, in the absence of SR

This condition of simulation, though unrealistic, allows one to evaluate the influence of P-sites *per se* on the kinetics of the excited state. If calcium were injected with no P-sites present, even on the troponin, the system would rapidly approach a new equilibrium with a higher T–Ca concentration. As more P-sites are added, the initial rapid increase in T–Ca is hardly affected. However, as the P–Mg dissociates more calcium is bound to the P-sites and the 'relaxation' of T–Ca is more rapid and complete.

The time course of the concentrations of the various complexes of P-sites and T-sites with Ca^{2+} and Mg^{2+} is illustrated in Fig. 1 for $[\Sigma P] = 840\ \mu M$, as found in frog muscle.

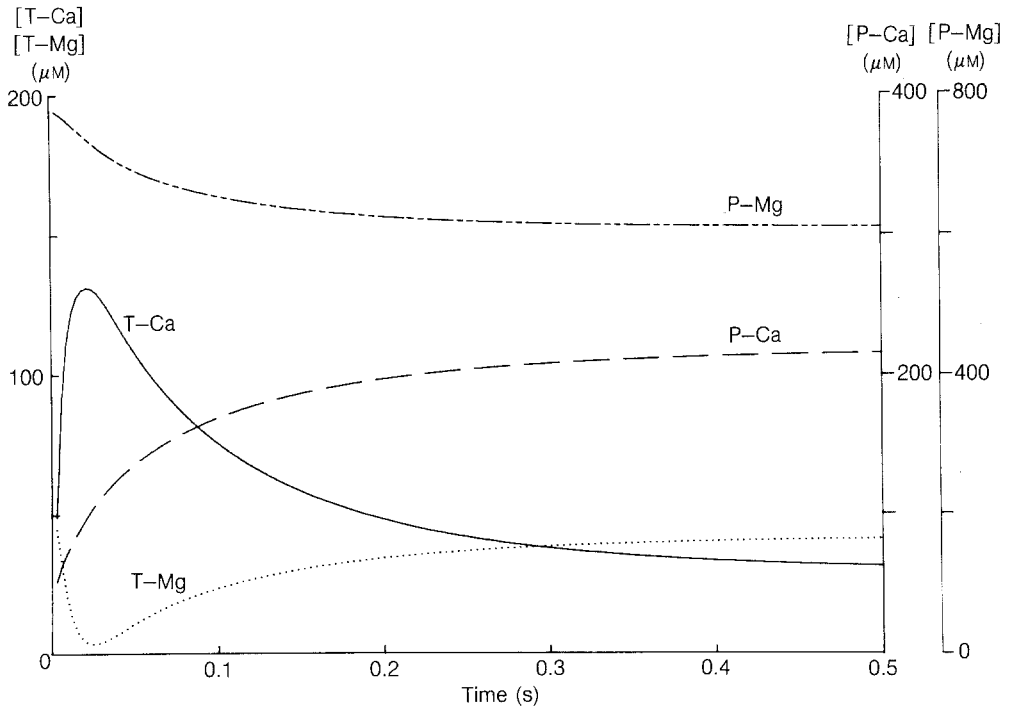

Fig. 1. Time course of the T- and P-sites complexes with Ca^{2+} and Mg^{2+} in response to a Ca^{2+} pulse (200 μmol l^{-1}) at $t = 0$. No SR activity. P-site concentration is 840 μM, as in frog muscle. For starting conditions, see Table 2.

The T–Ca concentration increases rapidly to reach a maximal value of 134 μM (94% saturation) after 20 ms. This is produced by Ca^{2+} binding to the apo T-sites and also by replacement of Mg^{2+} by Ca^{2+} on the T–Mg complexes, the concentration of which diminishes accordingly (from 52 μM to 3 μM). Relaxation begins at 23 ms and the T–Ca concentration is reduced to 70 μM (50% saturation) after 120 ms. At the end of the simulation, it has fallen to 32 μM. During this time T–Mg has returned to 41.2 μM.

The free Ca^{2+} transient caused by the Ca^{2+} pulse (Fig. 2, curve a), increases to 13.5 μM in 15 ms; this occurs before T–Ca reaches its maximal value and returns to around 1 μM, after 50 ms, when T–Ca saturation is still very high (77%).

By contrast with the rate of T–Ca formation, the P–Ca concentration increases much more slowly: its initial rate is four times slower, as expected from the ratio of 4 to 1 between the apo-T- and apo-P-site concentration at the start (Table 2). After saturation of the apo-P-sites, P–Ca formation continues at the expense of the P–Mg complexes, the dissociation of which becomes the rate-limiting factor of the P–Ca formation. At the end of the run, [P–Ca] has reached 208 μM. Thus, P-sites have eventually gained 168 μmol of Ca^{2+}, that is, they have absorbed 84% of the Ca^{2+} pulse.

It is interesting to note here that the binding of Ca^{2+} to T- and P-sites previously occupied by Mg^{2+} produces a small (7%) increase in free Mg^{2+} concentration.

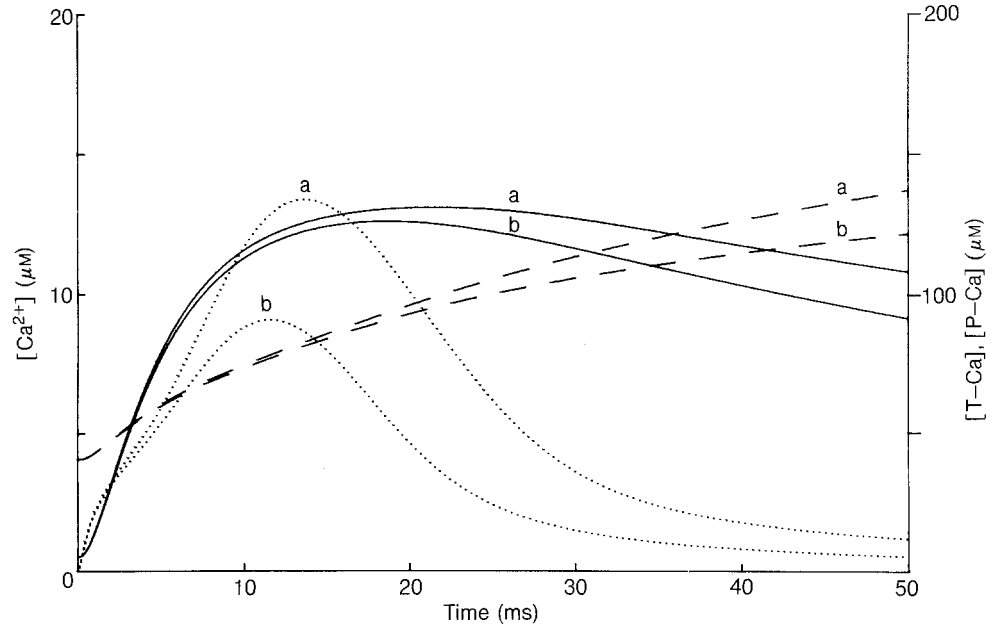

Fig. 2. Time course of [T–Ca] (———); [P–Ca] (– – – –) and free [Ca^{2+}] (. . . . .) during the first 50 ms of the simulation. a, only T- and P-sites, no SR activity. b, complete system as in Fig. 5, with 'high' SR activity. In both cases, the shape of the Ca^{2+} pulse is the same (see text: The model and its components).

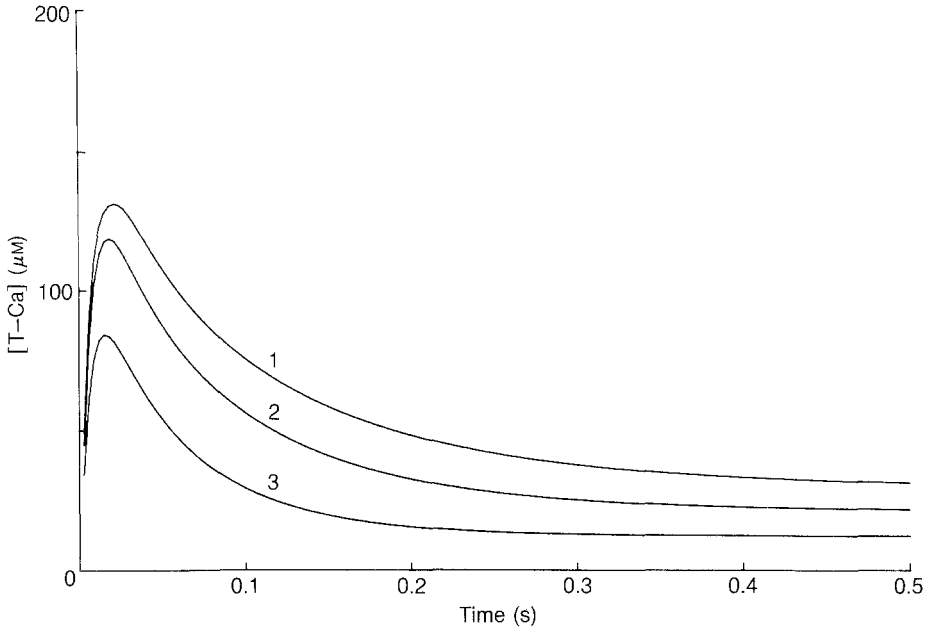

Fig. 3. Time course of T–Ca concentration after a Ca^{2+} pulse, for three concentrations of P-sites (μM): 1:840; 2:1140; 3:3140 (see Table 1). No SR activity.

As shown in Table 1, some fish muscles have a higher parvalbumin content than amphibian muscles. Simulations show that increasing the P-site concentration has four main results. These are illustrated in Fig. 3, which consists of superimposed traces of the time course of [T–Ca]: (1) the maximal T–Ca concentration is reduced [in the extreme case of the swimbladder muscle to 86 μM (61% saturation)]; (2) relaxation starts earlier; (3) the relaxation rate is increased; and (4) the final T–Ca concentration is lowered, that is, a larger fraction of the Ca^{2+} pulse is eventually bound on P-sites.

The relaxing effect of P-sites thus increases with their concentration. When the latter is very high, full activation is also prevented by a significant competition between T- and P-sites for calcium.

Relaxation controlled by the sarcoplasmic reticulum – no parvalbumin (P-sites from troponin only)
When the SR activity is 'high', that is, 700 μmol Ca^{2+} l^{-1} s^{-1} (see Table 1), relaxation is complete by the end of the run. The main effect of the absence of parvalbumin is to increase considerably the time during which the T-sites remain 95% saturated from 26 to 80 ms (Fig. 4, trace 1). This prolongation of the T–Ca saturation is directly related to a much higher and longer Ca^{2+} transient than in the presence of parvalbumin: the free Ca^{2+} concentration reaches 37.5 μM after 20 ms and is still at 14 μM after 50 ms.

Since the SR calcium pump rate is strongly influenced by temperature (Q_{10} = 3), the kinetics of [T–Ca] have been examined at three different levels of SR activity as defined

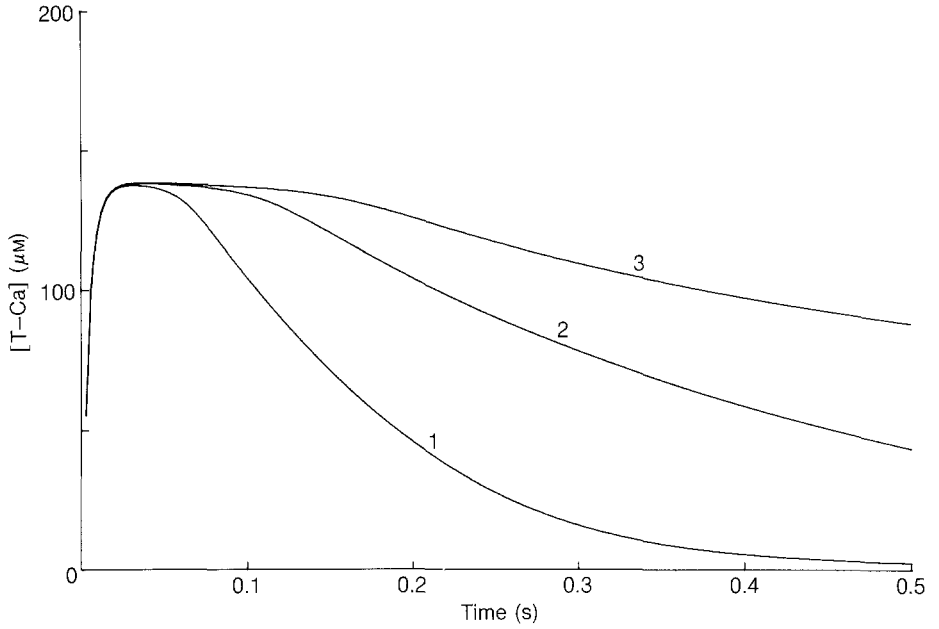

Fig. 4. Evolution of [T–Ca] after a Ca^{2+} pulse, for different SR activities and in the absence of parvalbumin (P-sites from troponin only). V_{max} for the SR (μmol Ca^{2+} l^{-1} s^{-1}): 1, 700 ('high'); 2, 235 ('medium'); 3, 78 ('low').

by their V_{max} values: 'high', 'medium' and 'low': 700, 235 and 78 μmol Ca^{2+} l^{-1} s^{-1}, respectively. These values are chosen to mimic SR activity at three different temperatures, with intervals of 10° C between each.

As illustrated by the superimposed traces for the time course of [T–Ca] in Fig. 4, 'relaxation' is much impaired as the SR activity is reduced. At 'low' activity, relaxation is so slow that twitching once per second would not be possible.

The complete system of T-sites, P-sites and SR

The complete system can now be evaluated as it exists in muscle and the relative contribution of SR and of parvalbumin to relaxation determined. The results in Figs. 5 and 2b show the time course of the system for frog muscle, at 'high' SR activity ($\Sigma P = 840\ \mu M$, $V_{max} = 700\ \mu$mol Ca^{2+} l^{-1} s^{-1}).

The formation of T–Ca is essentially unaffected by the presence of the SR. In contrast, the rate of relaxation is accelerated: half relaxation is now obtained in 78 ms, and full relaxation ([T–Ca] = 5.2 μM) after 375 ms, though in the absence of the SR, [T–Ca] is still about 30 μM after 500 ms.

The highest cytoplasmic free Ca^{2+} concentration in this 'realistic' simulation remains below 10 μM (Fig. 2, curve b), though in the absence of SR activity it reaches 13.5 μM, with the same pulse parameters. This indicates that the SR activity together with the

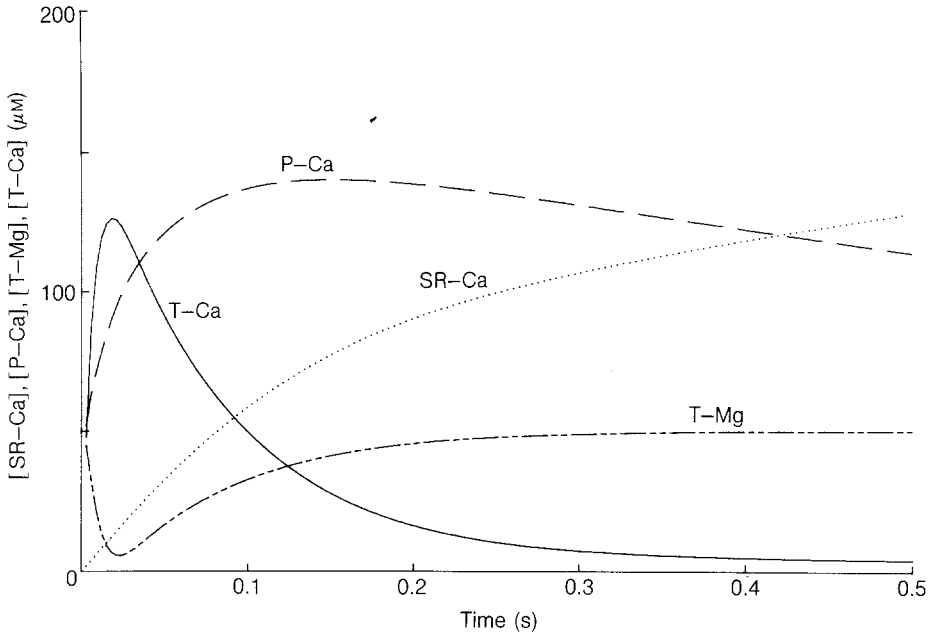

Fig. 5. Simulation of the response of P- and T-sites to a Ca^{2+} pulse, in the presence of the SR pump ('high' activity, see Fig. 3). SR–Ca denotes the amount of Ca^{2+} taken up by the SR. P-site concentration: 840 μM.

Ca^{2+} binding proteins influences the Ca^{2+} transient. As already found in the example for simulation without SR activity (Fig. 2a), the maximal value of free [Ca^{2+}] precedes the highest T–Ca concentration and declines to 0.5 μM when T–Ca is still 66% Ca^{2+} saturated. This behaviour is reminiscent of the original observation of Ashley & Ridgway (1970) and Ashley *et al.* (1974) who found that the aequorin signal reached its maximum before the peak of twitch tension, and returned close to its resting level before relaxation was complete.

The formation of P–Ca is now biphasic; it increases during the first 150 ms or so and then decreases during the second half of relaxation, as Ca^{2+} is gradually removed from the system by the effect of the SR pump. This is the behaviour expected from the hypothesis of Gillis & Gerday (1977) where parvalbumin is seen as a temporary Ca^{2+} store interfaced between the myofibrils and the SR.

As the SR activity is reduced, the time course of relaxation changes as illustrated in the Fig. 6. The first part of relaxation is little affected, while the last part becomes slower and incomplete. However, by comparing Figs. 4 and 6, it can be seen that the presence of parvalbumin permits relaxation to occur despite the effect of the reduced rate of SR pumping. These numerical calculations indicate how the Ca^{2+} lost by the T-sites and from the cytosol, is redistributed between both the P-sites and the SR when half relaxation was achieved. The relative contribution to relaxation by the two systems is pre-

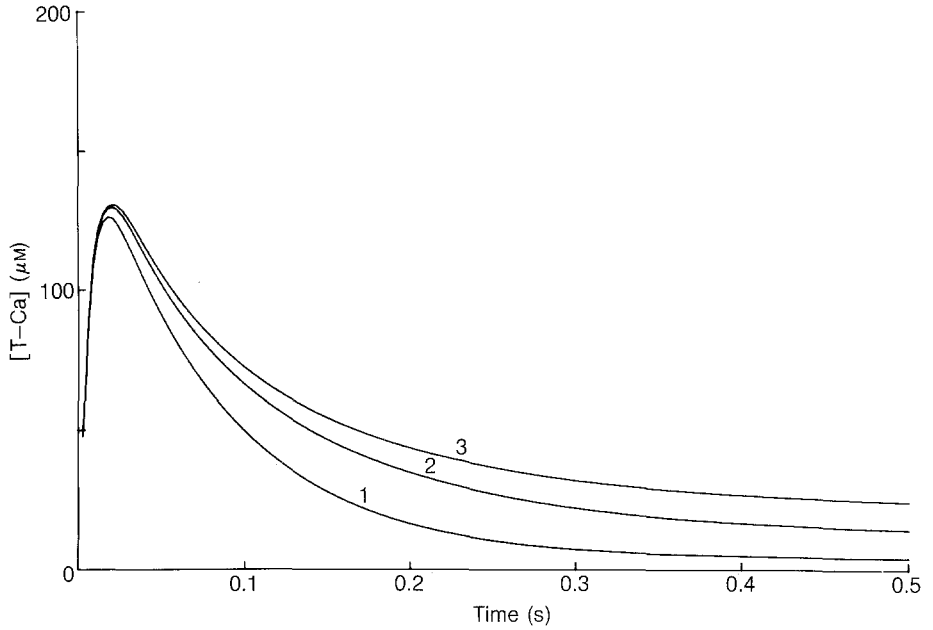

Fig. 6. Time course of [T–Ca] after a Ca^{2+} pulse, in the presence of parvalbumin (P-sites, 840 μM) and at three different SR activities: 1, 'high'; 2, 'medium'; 3, 'low' (see Fig. 4).

sented in Fig. 7. As the pumping rate of the SR is reduced, the first part of relaxation is increasingly determined by Ca^{2+} binding to P-sites.

The return of the [T–Ca] and [P–Ca] to their very low resting values is achieved by the SR, which is the ultimate means of reducing the Ca^{2+} content of the system. The time courses, however, are very different. As shown in Fig. 5, when relaxation is

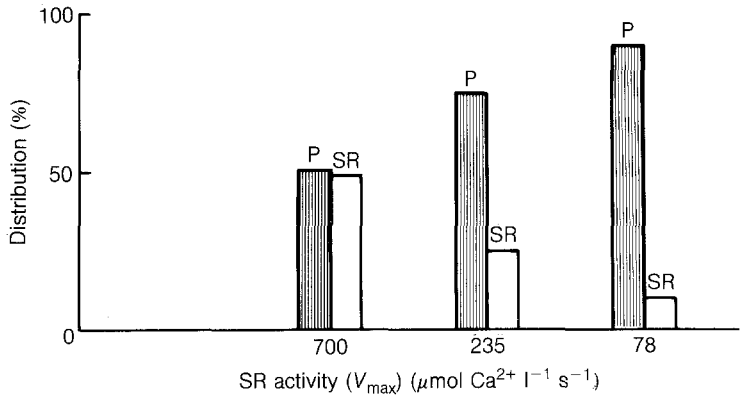

Fig. 7. Distribution (%) between P-sites and the SR of the Ca^{2+} removed from T–Ca and the cytosol during the first half of relaxation, at three levels of SR activity. (Constructed from the numerical data of the simulations.)

complete, the P–Ca concentration is still well above the resting level. This is due to the much smaller off rate constant of Ca^{2+} from P-sites than from T-sites, and the fact that the SR pump cannot operate at its maximal rate at the low $[Ca^{2+}]$ present during the second part of relaxation.

Repetitive stimulation is another way to study the relative importance of parvalbumin and the SR in controlling contraction. Here the important factor is the completeness of relaxation before the next pulse in order to avoid or to delay a progressive increase of the [T–Ca].

At a stimulation frequency of 2 pulses s^{-1}, the results of Fig. 4 predict that the presence of parvalbumin is not required to prevent tetanus, provided that the SR activity is 'high' for, in this case, complete relaxation is obtained in less than 0.5 s. The simulation illustrated in Fig. 8a confirms this expectation for a system containing no parvalbumin, that is, P-sites from troponin are included. The ability to contract repetitively without tetanus occurring depends on the frequency of stimulation and upon SR activity. If the latter is reduced by three times ('high' to 'medium' tetanus quickly develops, for at the third pulse both T- and P-sites are Ca^{2+} saturated (Fig. 8b). The effect of parvalbumin under these more critical conditions is that the higher the P-site concentration, the longer the delay before tetanus occurs. At the parvalbumin concentration existing in frog muscle ($\Sigma P = 840\ \mu M$), its presence delays the onset of tetanus until the ninth pulse and, at the very high P-site concentration occurring in toad fish swimbladder muscle ($\Sigma P = 3.14$ mM), tetanus is considerably delayed (Fig. 8c), not occurring until the fifteenth excitation (not shown). However, tetanus would inevitably occur if stimulations were continued and relaxation is less and less complete as the P–Mg store is used up and transformed into P–Ca.

Discussion

The resting state

From the results of Table 2, three points emerge. (1) About 30% of the so-called Ca^{2+} specific sites (T-sites) are occupied by Mg^{2+}, at the free Mg^{2+} concentration present in the cytosol. This point has not been appreciated before. It affects the kinetics of the T–Ca formation, and may affect the rate of contraction if the on rate constant for T–Mg used here is confirmed by direct experimental determination. (2) More than 90% of the P-sites are loaded with Mg^{2+}. This situation will link the rate of P–Ca formation to the rate of P–Mg dissociation. (3) A small fraction (about 2.5%) of the P-sites exists in the metal free or apo-form. In muscles with a very high parvalbumin content, the apo-P-site/apo-T-site ratio approaches unity. Therefore a direct competition for Ca^{2+} is expected. The expectations from the last two points have been confirmed in the simulations of the excited state (Fig. 3).

Evaluation of the model of the excited state

The constraints of the model, that is, no diffusion delays or co-operativity, indicated in

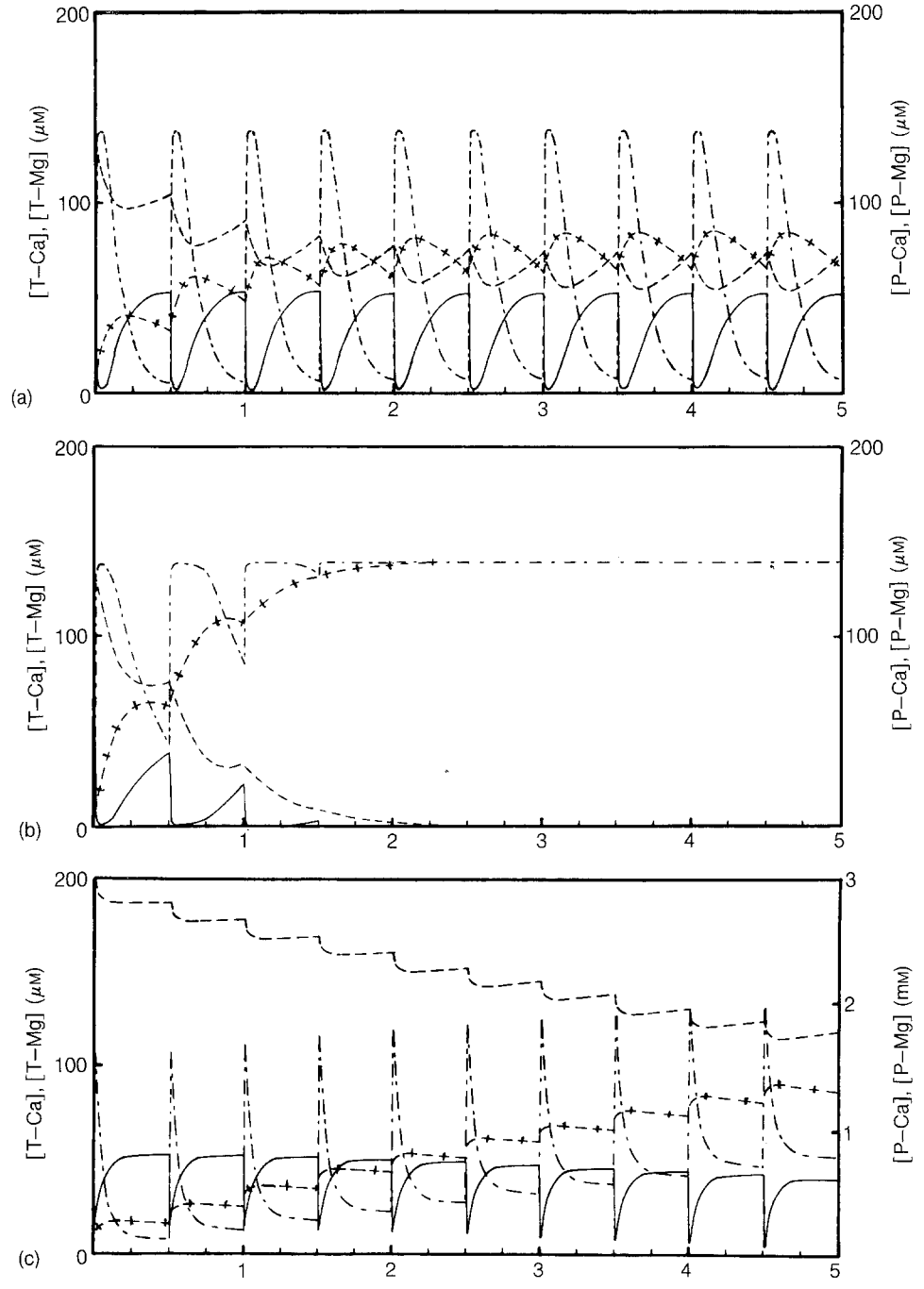

Fig. 8. Simulation of repetitive stimulations: 10 Ca^{2+} pulses of equal amplitude (200 μmol Ca^{2+} l^{-1}) 'injected' into the system at 2 s^{-1}. The traces give the T- and P-sites complexes with Ca^{2+} and Mg^{2+}. (a) SR activity 'high', no parvalbumin; (b) same as (a) but the SR activity reduced to 'medium'; (c) same as (b), but with a P-site concentration (3.14 mM). Traces: (–·–·–·) T–Ca; (———) T–Mg; (+ –+ –+ –+ –) P–Ca; (– – – – –) P–Mg.

the description of the model together with the binding constants (Table 1) are the same, in essence, as those used by Robertson *et al.* (1981). A major difference between the two studies concerns the way in which Ca^{2+} is 'injected' into and removed from the system. Robertson *et al.* (1981) force the free $[Ca^{2+}]$ to 'rise' and 'fall' by an empirical equation, the constants of which are adjusted to start from 10^{-8}M, attain 6.3×10^{-6}M and return to the resting level in 50 ms. They thus treat the free $[Ca^{2+}]$ transient as an independent variable in the sense that it does not depend upon the characteristics of the SR pump or upon the Ca^{2+} binding by T- or P-sites. An alternative adopted in the present paper is that Ca^{2+} is injected as a fixed quantity and the various Ca^{2+} binding sites as well as the SR are allowed to influence free $[Ca^{2+}]$ according to their own characteristics. The time courses obtained of the activation–relaxation cycle and of the free $[Ca^{2+}]$ transient mimic the characteristics of a frog muscle twitch closely enough to make our model seem satisfactory.

The difficulty in modelling repetitive stimulations correctly has also been discussed. Our results differ from those observed with live muscle: here, tetanus is calculated to occur after about 10 pulses, at a stimulus frequency of two per second and at the SR activity corresponding to 15° C, while frog muscles sustain long series of 'unfused' twitches when stimulated under these conditions. However, there is a critical relation between stimulation frequency and SR Ca^{2+} pump activity on the approach to the tetanic state and parvalbumin affects this relation. Obviously, the model will have to be re-evaluated when more detailed information becomes available about Ca^{2+} pulses and SR activity under conditions of repetitive stimulation.

Kinetics of the activation–relaxation cycle

Formation of the T–Ca complex, that is, muscle activation, is not prevented by a high concentration of parvalbumin, because most of these binding sites are already occupied by Mg^{2+}. Replacement of Mg^{2+} by Ca^{2+} is practically limited by the rate of P–Mg dissociation which is slow compared with the rate of T–Ca formation. This point confirms one conclusion of the Robertson *et al.* (1981) study. It also eliminates a suggestion made by some authors that parvalbumin is concentrated inside the SR in order to avoid interference with muscle activation (Homsher & Kean, 1978).

When the interaction between troponin and parvalbumin are modelled separately, the latter can produce the dissociation of the T–Ca complex within a few hundred milliseconds, that is, in the duration of a twitch of amphibian and fish muscles. However, this relaxing potential of parvalbumin depends on its concentration and this is by far the most important difference between the study of Robertson *et al.* (1981) and the present one. Robertson *et al.* (1981) examined the situation in mammals, where the contraction time is very short and the parvalbumin content is very low (70 μM, and similar to that of troponin). We modelled the situation in lower vertebrates, where the parvalbumin concentration is much greater: 350 μM and above. At a low parvalbumin concentration, we agree with their conclusion that the relaxing effect is negligible. On the other hand, at the high concentration found in fishes and amphibians, our study

clearly shows that parvalbumins can contribute to relaxation in the 200 ms following stimulation.

The purpose of this study was also to estimate the relative contribution of parvalbumin and the SR to muscle relaxation. Parvalbumin influences the early part of the contraction cycle. First, by trapping a part of the Ca^{2+} pulse on the apo-parvalbumin molecule. This effect would have little influence on muscle activation, except at extremely high concentrations of parvalbumin. Secondly, parvalbumin causes the dissociation of the T–Ca complex, so that by the time the T–Ca concentration has dropped to 70 μM (50% saturation) at least one half of the Ca^{2+} removed from T-sites and the cytosol is bound onto parvalbumins. The kinetics of this phase is governed by the off rate constant of Mg^{2+} from P–Mg, which was taken here as 2.7 s^{-1} (Potter *et al.*, 1978). The new determination of the rate constants by the same group (Robertson *et al.*, 1981) indicate that relaxation could even be faster than reported here. The influence of the SR dominates the second half of relaxation and is essential to restore the very low free Ca^{2+} concentration and [T–Ca] of the resting state, as well as to dissociate the P–Ca complexes.

Our stimulations also show that, at the time relaxation is complete (that is, [T–Ca] has returned to the resting level), a large fraction of the Ca^{2+} pulse is still bound to parvalbumin and should return to the SR during the post-contractile period (recovery). This result may provide a new way of interpreting the translocation of Ca^{2+} during the recovery phase observed by Winegrad (1968) using ^{45}Ca autoradiography, where for a few seconds after a tetanus, calcium was present mainly in the intermyofibrillar space, instead of, as at rest, in the terminal cisternae of the SR. Winegrad assumed that Ca^{2+} was located into the longitudinal tubules of the SR and from there, was slowly displaced towards the terminal cisternae. Our results indicate, on the other hand, that this intermyofibrillar calcium is bound to parvalbumin, freely present in the cytoplasm (Gillis *et al.*, 1979). Recently, Somlyo *et al.* (1981) arrived at the same conclusion from their localization of Ca^{2+} using the electron microprobe technique. Parvalbumin thus appears to act as a temporary calcium store, which is slowly depleted during recovery. As already suggested by Curtin & Woledge (1978) this situation may be connected to the question of the 'unexplained heat' of muscle energetics. A fraction of the heat produced during a tetanus cannot be accounted for by the known amount of ATP and phosphocreatine broken down. In frog muscle for a 1 s tetanus, the discrepancy amounts to 20–40 J l^{-1}. Closset & Woledge (in Curtin & Woledge, 1978) have determined that the binding of Ca^{2+} to parvalbumin liberates about 27 KJ per mol Ca^{2+} bound, determined in the presence of a physiological concentration of free Mg^{2+}. If, at the end of a short tetanus, there is still 100–200 μmol of Ca^{2+} l^{-1} attached to the P-sites (Fig. 8b), one might expect an 'extra heat' output of about 2.7–5.4 J l^{-1}, with no concomitant splitting of ATP. This may represent 14% of the 'unexplained heat'.

The effect of reducing the SR pumping rate upon relaxation has also been examined and to what extent parvalbumins could compensate for this reduction. For a single stimulus, the presence of parvalbumin, at the concentrations used here, can ensure a nearly normal relaxation even when the SR activity has been reduced by about

10 times. However, in the repetitive stimulations studied, it is clear that parvalbumin can compensate for a reduced SR pump only for short periods of contraction. Otherwise, tetanus would occur inevitably as the Ca^{2+} binding capacity of parvalbumin is depleted.

The physiological role of parvalbumins

It is essential to consider the species and tissue distribution of parvalbumin when discussing its physiological role. The main fact is that a high content of parvalbumin is found only in fast skeletal muscle of fish and amphibians. These animals, in contrast to mammals, live in a wide range of external temperatures and hence, since they are poikilotherms, have a wide range of internal temperatures, especially in temperate and arctic regions. It has been proposed (Gillis, 1980) that the specific role of parvalbumins is to allow fast relaxation even when the SR activity has been reduced by a drop in temperature. The study reported here confirms the plausibility of this hypothesis, but restricts its applications to short bursts of muscle activity, like the quick movements of attack or escape. In this context, it may be relevant to note that during slow 'cruising' swimming, fish use primarily their red muscle (Bone, 1966) which has a low content of parvalbumin, while during fast, 'burst' swimming, they use their white muscle in which the parvalbumin concentration is fairly high (Hamoir, 1974). In their predator–prey activities, it would be adaptive to swim very rapidly for brief periods. To accomplish this, in water temperature ranging from 1–20° C, it may be easier to evolve muscle with a high parvalbumin content than to increase the V_{max} of the Ca-ATPase necessary to function effectively at reduced temperatures.

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