# Distribution of Calcium During Contraction and Relaxation of Crayfish Skeletal Muscle Fibre

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**Abstract.** A model of activation of muscle contraction has been applied to the crayfish isolated skeletal muscle fibre The model is based on calcium diffusion and binding to specific regulatory sites in a sarcomere Calcium ions activate interactions of contractile proteins and thus the generation of force The model quantifies the relation between calcium released from intracellular stores and force elicited Experimental tension records from isolated crayfish skeletal muscle fibres under voltage clamp conditions are analyzed Model parameters were determined either via approximation of the onset of tension by the model solution or from the model based relations between the tension maximum, and depolarizing pulse length and amplitude This allowed to determine time changes of free and bound calcium distribution in the sarcomere and the calcium release from terminal cisternae The steady state calcium concentration at terminal cisternae showed S-shaped voltage dependence with saturation below approx 10 $\mu$mol/l at positive membrane potentials

**Key words:** Skeletal muscle fibre — *Astacus fluviatilis* — Contraction — Model — Calcium distribution — Calcium release

## Introduction

The main difference in EC coupling between invertebrate and vertebrate skeletal muscle follows from the type of electrogenesis in the surface membrane systems In contrast to the sodium action potential in vertebrates, the calcium action potential may play a role in direct activation of contraction in invertebrates, e g by the mechanism of calcium-induced calcium release from the sarcoplasmic reticulum (Zacharová and Zachar 1967, Zachar 1981) Muscle fibers of the crayfish, however, possess all basic components which have recently been invoked as key elements of EC coupling in vertebrate skeletal muscle (voltage sensor, T-SR junction mediators) (Zachar and Zacharová 1989, Formelová et al 1990, Zacharová et al 1990) The diads in muscle fibers of *Astacus fluviatilis* show a similar feet structure as do triads

in vertebrate muscle (Uhrík et al 1984, 1986)

Elucidation of the actual mechanism of the excitation and contraction coupling requires the knowledge of the voltage dependence of calcium release from the terminal cisternae, of calcium distribution during contraction, and of its regulation role for calcium release and muscle fibre contraction

The hypothesis developed by Poledna (1987, 1989, 1991), and represented by the model, has included the essential mechanisms which determine the courses of activation and of relaxation of contraction This allows obtaining of otherwise nonmeasurable variables, such as the time course of calcium release and the time and spatial distribution of calcium in the sarcomere These are important for the analysis of regulation processes in the skeletal muscle cell

## Materials and Methods

The muscle fibre preparation and the voltage clamp apparatus have been described elsewhere (Poledna and Lacinová 1988) Briefly, isolated muscle fibers were dissected from m extensor carpopoditi of the crayfish *Astacus fluviatilis* One end of the tested intact fibre was isolated by two vaseline seals, which divided the chamber into three compartments The compartment between the seals was grounded to increase the virtual seal resistance The tested end of the fibre, of a length of about 0 5 mm from the tendon to the seal, was voltage clamped Current was applied into the compartment containing the rest of the fibre to control voltage in the test compartment The tension of the terminal segment of the fibre in the test compartment was measured isometrically The crayfish saline (van Harreveld) contained (in mmol/l) $Na^+$ 205 3, $K^+$ 5 4, $Ca^{2+}$ 13 5, $Mg^{2+}$ 5 6, $Cl^-$ 248 8, $Tris^+$ 5 In the current compartment, $Ca^{2+}$ was omitted, and the crayfish saline in the grounded compartment was modified by partially replacing $Na^+$ ions by 100 mmol/l $TEA^+$ Measurements were performed at temperature in the range 10–12 °C

### *Data processing*

Voltage, current and tension signals from the voltage clamp apparatus were displayed on a Tektronix 11201 Digitizing Oscilloscope The oscilloscope was interfaced to personal computer via a GPIB interface, where data were processed Data were approximated by the model developed by Poledna (1989), as briefly described below

### *Model*

Calcium ions are released from terminal cisternae This event is controlled by tubular membrane voltage Calcium ions bind to specific troponin C regulatory sites Due to the high binding rate constant, the calcium flux is able to temporarily fill them, even though the equilibrium concentration is below the amount required to occupy these sites (Johnson et al 1981) Also, Ca ATPase has the same binding rate and contributes to the concentration of fast binding sites This means that released calcium immediately occupies the nearest free fast binding sites and subsequently continue further Saturated binding sites form a region, which expands longitudinally towards the center of the sarcomere Only in this region can free calcium diffuse, as expressed by the diffusion equation

$$\partial c/\partial t = D \ \partial^2 c/\partial x^2 \quad c(0,t) = c_0 \quad c(x,0) = 0 \tag{1}$$

Ions, crossing the region boundary cannot diffuse because they are immediately bound to the fast binding sites. This process expands the region. The movement of the region boundary is described by the equation

$$x_h(t) = k \cdot t^{0.5} \tag{2}$$

being the consequence of equation (1) at $D = 0$ outside the region with saturated sites (Crank 1964; Poledna 1989).

Relation of the parameter $k$ to the steady calcium concentration at terminal cisternae, $c_o$, and to the fast calcium binding sites concentration, $c_x$, was obtained from the solution to equation (1) in the form (Poledna 1989)

$$G(k/(2 \cdot D^{0.5})) = c_0/c_x - 1 \tag{3}$$

where the function $G$ is defined as

$$G(z) = \pi^{0.5} \cdot z \cdot \exp(z^2) \cdot \mathrm{erf}(z)$$

Cisternae form diads with tubules at the A–I band border. The calcium release sites are on terminal cisterna membranes in the area of the tubulo-reticular junction. Therefore, the origin $x = 0$ is at the A–I boundary and the calcium saturated region expands from this point symmetrically to both sides along the fiber axis.

The calcium flux is given by the Fick's law and

$$J(x,t) = -2 \cdot D \cdot \partial c/\partial x \tag{4}$$

At point $x = 0$, there must be total calcium flux from the terminal cisterna. From the solution to equation (1) we get (Poledna 1989) the flux in a halfsarcomere

$$J(0,t) = c_x \cdot k \cdot \exp(k^2/(4 \cdot D)) \cdot t^{-1/2} \tag{5}$$

with the total amount released

$$M(t) = 2c \cdot c_x \cdot k \cdot \exp(k^2/(4 \cdot D)) \cdot t^{1/2} \tag{6}$$

Only those parts of myofilaments which are in the space with calcium occupied specific troponin C sites can form bridges and generate force. This can be expressed by the differential equation

$$\mathrm{d}m/\mathrm{d}t = p \cdot (\beta \cdot k \cdot t^{0.5} - m) \tag{7}$$

where $p$ is the binding rate of myosin heads, $m$ is the number of created bridges and $\beta$ is the number of myosin heads in the length unit. The first term in brackets is the number of heads able to form bridges. The solution to equation (7) can be expressed in the form of infinite series

$$m = \beta \cdot k \cdot t^{0.5} \sum_{i=1} a_i s^i \tag{8}$$

with the coefficients given by the recurrent formula

$$a_i = -a_{i-1}/(2i + 1); \quad a_1 = 1/3$$

where $s = 2pt$. The series in formula (8) converges and only 10 terms are sufficient for a good approximation of experimental records.

To obtain the parameter $k$ of the solution to equation (1), two methods were used. The first one is based on the least square method approximation of a tension record by solution (8) to equation (7). The onset of isometric tension $T(t)$ should be proportional to $m(t)$. The coefficient is

$$T(t)/m(t) - T_{max}/(l_{1/2} \cdot \beta) \tag{9}$$

$T_{max}$ is a maximal amplitude of contraction, where saturation of all fast binding sites can be supposed, and $l_{1/2}$ is the length of filaments overlap in a halfsarcomere. $\beta$ is canceled from equation (8) by this coefficient. Parameters $p$ and $k$ are obtained. The most important parameter $k$ characterizes the region where actin and myosin filaments interact. It can be used to determine steady state calcium concentration at terminal cisternae according to equation (3), calcium flux from terminal cisternae (5), and total amount of calcium released (6).

The second method approximates the maxima of tension amplitude $T(V, t)$, as a function of pulse amplitude, $V$, and width, $t$, by the equation

$$T(V, t) = a(V) \cdot t^{0.5} \tag{10}$$

which is related to equation (2) by

$$k(V) = l_{1/2}/T_{max} \cdot a(V) \tag{11}$$

It is supposed that a super-threshold depolarizing pulse saturates regulatory sites in a part of the sarcomere. The tension maximum, $T(V, t)$, is proportional to the saturated part of the sarcomere. When the saturated region covers the whole overlap of myosin and actin filaments, all heads can form bridges and the generated force reaches a maximum, $T_{max}$.

## Results

The onset of tension was approximated (Fig. 1) by the model according to equation (8). Parameters $k$ and $p$ were determined by the least square method. Parameter $p$, which corresponds to the binding rate of myosin heads to actin sites, was $47.4 \pm 0.9$ $s^{-1}$ ($n = 20$). It was almost depolarization independent.

Parameter $k$ characterizes the expansion of the region with calcium saturated specific troponin C sites. This parameter is voltage dependent and measurements from 4 fibers are shown in Fig. 2. The voltage dependence saturated at a membrane potential above +10 mV. Since approximation required a good resolution of tension records, the fibre was stretched to optimal length for tension measurements, i.e. to 1.2 of its slack length. Under these conditions, the parameters of filament overlap are $3.95 \pm 0.8$ $\mu$m and $10.5 \pm 0.3$ $\mu$m for the A band and sarcomere length respectively (Zachar and Zacharová 1966).

Equation (2) offers an independent possibility to determine parameter $k$. Contractile responses to rectangular depolarizing pulses of different amplitudes and durations were measured. In accordance with equation (2), the maximal amplitude

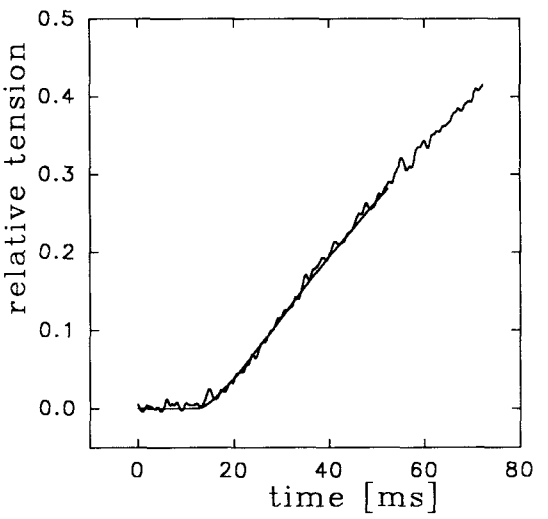


**Figure 1.** The experimental record of tension onset and its approximation by the model. The crayfish skeletal muscle fibre was voltage clamped at 0 mV. The model parameters are $p = 50.1\ s^{-1}$, $k = 4.16\ \mu m \cdot s^{-1/2}$.

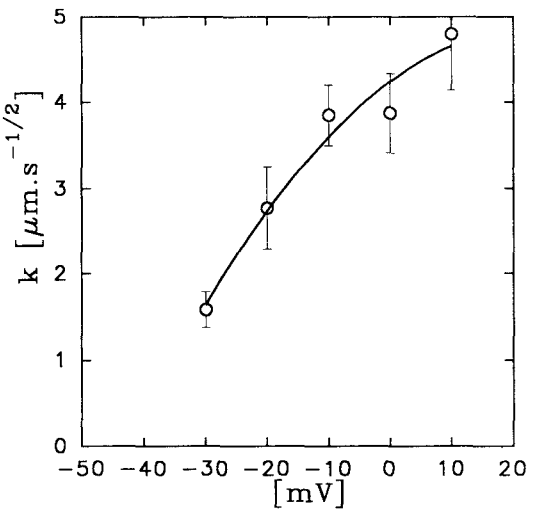


**Figure 2.** The voltage dependence of the model parameter $k$, which describes calcium distribution in the sarcomere determined by approximation of the tension onset. Its relation to the steady state free calcium concentration at terminal cisternae is given in Table 1.

of contraction, which is approximately proportional to the width of the region of saturated calcium sites, depends on the square root of the pulse duration (Fig. 3).

Moreover, based on the model we can use time and voltage dependencies of the contraction amplitude to determine the voltage dependence of parameter $k$ (Fig. 4). Parameter $k$ is related to the steady state calcium concentration at the tubulo-reticular junction. This relation is expressed by equation (3). To determine this steady state concentration, it is necessary to know the concentration of fast

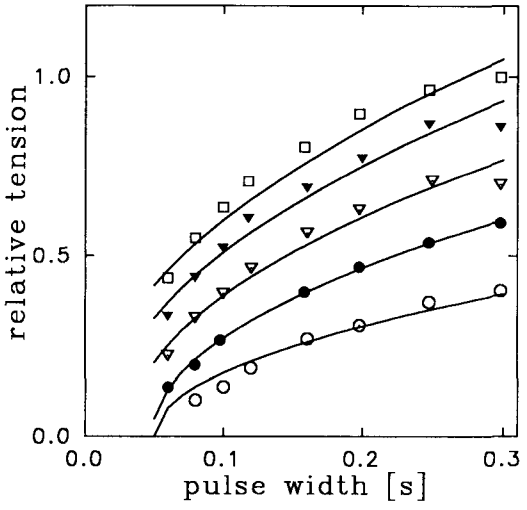


**Figure 3.** Parabolic approximations of tension maxima for different lengths and amplitudes of depolarizing voltage clamp pulses. The symbols represent following membrane potentials: □ 0 mV, ▼ −10 mV, ▽ −20 mV, • −30 mV, o −40 mV.

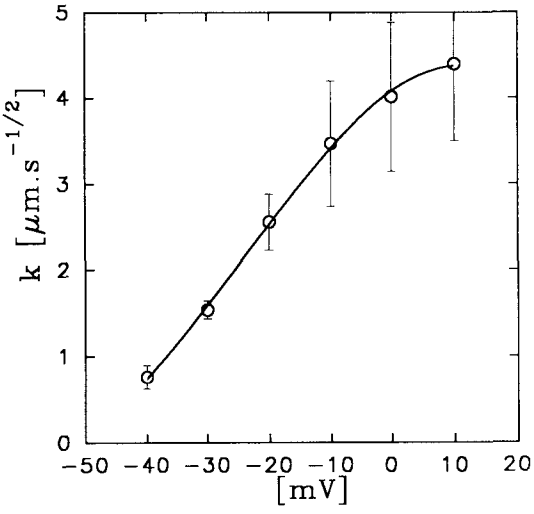


**Figure 4.** The voltage dependence of the model parameter $k$, which describes calcium distribution in the sarcomere determined by approximation of tension maxima. The relation to the steady state free calcium concentration at terminal cisternae is given in Table 1.

calcium binding sites and the diffusion coefficient. These values have not been determined experimentally. Some rough estimates are possible.

The concentration of fast binding sites, $c_x$, can be estimated from literary data Due to different spatial arrangements of actin and myosin filaments (Zachar 1971), the density of actin filaments in the crayfish fibre is about two times higher than that in the frog fibre. Invertebrate troponin, in contrast to vertebrate troponin, contains only one Ca-specific site (Wnuk et al. 1984). Therefore, the concentration

of troponin C specific calcium binding sites could be about 240 $\mu$mol/l.

Concentration of Ca binding sites on Ca ATPase can be estimated from their functional characteristics. For long super-threshold depolarizing pulses all troponin C binding sites are saturated. It means that there is about 240 $\mu$mol/l Ca bound. Besides Ca ATPase binding sites this represents the substantial part of calcium in the sarcoplasm. Parvalbumin, a calcium-binding protein of vertebrates which is soluble in the myoplasm, presumably acts as a soluble relaxing factor that traps calcium released from troponin. Soluble calcium-binding proteins which possibly are homologous to parvalbumins have also been found in the myoplasm of crayfish muscle (Cox et al. 1977). In barnacle fibers, closely related to the crayfish skeletal muscle, the concentrations of parvalbumins and related molecules are very low (less than 6 $\mu$mol/l (Griffiths et al. 1988; 1990)) and can be neglected in our estimation.

The time constant of relaxation determined from 4 fibers was 1.82±0.05 s. The slowest step of the calcium uptake is the Ca pump turnover, which is about 5 $s^{-1}$ at 22°C(Martonosi and Beeler 1983), i.e. approx. 2 $s^{-1}$ at 12°C. Other steps are much faster: the dissociation of calcium from troponin C is about 0.12 $ms^{-1}$ (Cannell and Allen 1984) and binding to the pump is about 0.35 $\mu mol^{-1} \cdot l \cdot ms^{-1}$ (Inesi 1981). Therefore, the relaxation of contraction can be described by a simplified model. Due to the slow rate of relaxation, reactions of calcium dissociation from troponin C and binding to Ca ATPase can be treated like being in the equilibrium state. For this part of relaxation the relative tension is proportional to troponin C occupancy

$$T = TC/T_0 = c/(K_{Td} + c) \tag{12}$$

and Ca uptake is described by

$$dc/dt + dTC/dt = -K_{Pa} P_0 \cdot r \cdot c \tag{13}$$

where $r$ is the pump turnover and $P_0 \cdot r$ is the availability of the pump calcium binding sites. The implicit solution to these equations is

$$T - \ln T - K_{Td}/T_0 \cdot \ln(T/(1-T)) = A + K_{Pa} \cdot K_{Td} \cdot P_0/T_0 \cdot r \cdot t \tag{14}$$

Approximation of the relaxation based on measurements in 4 fibers gave the value of 0.597 ± 0.048 $s^{-1}$ for the parameter at the variable $t$. This can be used to estimate the number of Ca pump specific binding sites $P_0$. Taking $T_0 = 240$ $\mu$mol/l, $K_{Pa} = 3.5$ $\mu mol^{-1} \cdot l$ (Martonosi and Beeler 1983), $K_{Td} = 1.0$ $\mu$mol/l (Potter and Zot 1982), and $r = 2$ $s^{-1}$ (5 $s^{-1}$ at 22°C, Martonosi and Beeler (1983)), $P_0$ is approximately 20 $\mu$mol/l.

Also, the calcium diffusion coefficient is important for quantitative estimation of parameters. Kushmerick and Podolsky (1969) compared the diffusion coefficients of ions and nonelectrolytes in aqueous solution and in muscle cell. They measured

a reduction by a factor of 2 for both nonelectrolytes and ions. They concluded that the diffusion of the ions is reduced by physical rather than chemical interactions. In contrast, the diffusivity of the calcium ion, which is taken up by the sarcoplasmic reticulum, is reduced from 700 $\mu m^2 \cdot s^{-1}$ to 14 $\mu m^2 \cdot s^{-1}$. Taking into account only physical factors, the calcium diffusion coefficient inside a muscle cell is expected to be $D = 350\ \mu m^2 \cdot s^{-1}$.

**Table 1**

| Membrane potential [mV] | $k$ $\mu m \cdot s^{-1/2}$ | SEM | [Ca] $\mu$mol/l | $k$ $\mu m \cdot s^{-1/2}$ | SEM | [Ca] $\mu$mol/l |
|---|---|---|---|---|---|---|
| -40 | – | – | – | 0.76 | 0.14 | 0.215 |
| -30 | 1.58 | 0.21 | 0.929 | 1.53 | 0.11 | 0.871 |
| -20 | 2.77 | 0.48 | 2.863 | 2.56 | 0.33 | 2.444 |
| -10 | 3.85 | 0.35 | 5.549 | 3.47 | 0.73 | 4.502 |
| 0 | 3.87 | 0.46 | 5.607 | 4.01 | 0.87 | 6.024 |
| 10 | 4.80 | 0.65 | 8.660 | 4.39 | 0.89 | 7.230 |

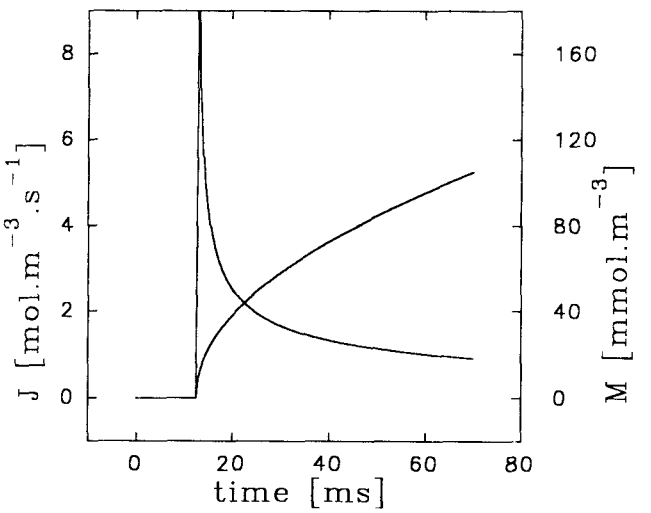


**Figure 5.** The modeled calcium flux from terminal cisternae, J, and total amounts of calcium released into the sarcoplasm, M, corresponding to the tension record shown in Fig. 1.

The diffusion constant $D = 350\ \mu m^2 \cdot s^{-1}$ and the total concentration of fast calcium binding sites, 260 $\mu$mol/l, can be used to estimate, by equation (3), the calcium concentration at terminal cisternae during calcium release. The concentrations of free calcium, $c_0 - c_x$, corresponding to measured values of parameter $k$, are shown in Table 1. Using the parameters measured, the model allows to determine also the time course of calcium flux from terminal cisternae and the total amount of calcium released (equations (5) and (6)). An example corresponding to the tension record from Fig. 1 is displayed in Fig. 5.

## Discussion

During activation of skeletal muscle, depolarization of the surface membrane propagates along the transverse tubular system and triggers calcium release from the sarcoplasmic reticulum via a mechanism which is not completely understood. This mechanism can be characterized by the relation between tubular membrane voltage and the amount of calcium released from the sarcoplasmic reticulum. There is a direct link between time courses of calcium release and contraction. The presented analysis of calcium release is based on the hypothesis (Poledna 1989) relating the number of switched-on crossbridges and elicited force to calcium occupancy of troponin.

Depolarization of a skeletal muscle fibre causes a transient increase in the myoplasmic free calcium concentration. This calcium transient is the net result of several simultaneous processes. Calcium is released from the sarcoplasmic reticulum and binds to a variety of different myoplasmic calcium binding sites. As a result, the flux of calcium through the sarcoplasm is expected to be much greater than the change in free calcium. Moreover, sharply localized release sites of calcium in tubuloreticular junctions and high concentration of fast calcium binding sites imply very nonhomogeneous calcium distribution. This was also confirmed by the detailed model of calcium diffusion and binding in the sarcomere (Hollý and Poledna 1989). The description of nonhomogeneous calcium distribution allows a more realistic explanation of the contraction mechanism.

In studying the activation of contraction, processes must be distinguished which can be treated as equilibrium processes (for instance, the late part of relaxation) and those far from equilibrium being described only as dynamic systems. This is the main reason why attempts to find a relation between average free calcium concentration in the sarcomere (measured by optical methods) and contraction have not been successful.

Ca ions are necessary for EC coupling in crayfish skeletal muscle fibers. Contraction is inhibited upon removal of Ca from external saline (Zacharová and Zachar 1967; Valko et al. 1967). A role of Ca in EC coupling is also supposed in vertebrate muscles. Even though membrane Ca currents do not appear to play any

role in the function of the voltage sensor (dihydropyridine (DHP) receptor), the voltage sensor has a site with chemical properties similar to those of the intrapore Ca-binding site of the Ca channels This site on the sensor has to be occupied to prevent inactivation (Ríos and Pizarro 1991) Pizarro et al (1989) demonstrated that all alkali ions substituted for Ca support EC coupling, that is, allow Ca release to occur There is morphological evidence (Block et al 1988) that only a half of Ca releasing channels is in direct contact with DHP receptors and the rest can be activated by calcium released through this voltage sensor controlled calcium release channels (Ríos and Pizarro 1988) This represents another role of calcium in EC coupling

It can be estimated from the calcium current measurements in crayfish skeletal muscle fibers (Henček and Zachar 1977, Henček and Zacharová 1991) that inward calcium current during depolarization is not able to saturate more than about 5% of troponin C specific regulatory sites, and Ca released from the sarcoplasmic reticulum is the essential source for contraction Therefore, Ca in the external solution is necessary for contraction in crayfish muscle either due to a higher selectivity of DHP receptor for Ca or due to the direct role of external Ca in calcium induced Ca release from the sarcoplasmic reticulum This question needs further elucidation of the function of different components of the calcium current (Henček and Zacharová 1991) in the crayfish muscle cell, since there is no direct relation between the total calcium inward current and tension For instance, a decline of calcium current at positive potentials is not reflected in tension changes (Zachar and Zacharová 1966, Poledna and Lacinová 1988)

The model does not allow the inward flux of extracellular calcium to be distinguished from the calcium flux from the sarcoplasmic reticulum The above estimates of Ca inward flux give a limit for their interference

The proposed hypothesis concerning nonhomogeneous calcium distribution during activation of contraction could explain the time courses of tension in different muscle fibers The description is a general one and it only requires that the calcium release occurs within a distinct site in the sarcomere Quantitative variations may arise from morphological differences, e g sarcomere length, position of a tubuloreticular junction (at Z-line or A-I border), thin and thick filament packing, number of specific regulatory sites on troponin C (influences concentration of fast binding sites), density of the sarcoplasmic reticulum, and density of calcium release channels The experimental application of the model requires simultaneous activation of all sarcomeres, i e under voltage clamp conditions

The model is not applicable e g to frog heart muscle cells, where the sarcoplasmic reticulum is scarce, calcium for contraction is supplied mainly from the extracellular space, and the assumption about an expanding calcium saturated region does not held

To study the isolated crayfish skeletal muscle fibre is interesting because of

its close relation to heart muscle as far as excitation contraction coupling and activation of tension are concerned. Mammalian heart muscle cells satisfy the above conditions and could be analyzed in the same way.

Ashley et al. (1991) reviewed models of the activation of muscle contraction by calcium, which used detailed reaction schemes. These models need time consuming computations and cannot be simply used in experiments. The presented model omits much of the known biochemical details of the crossbridge cycle but it suffices to provide a quantitative understanding of calcium action in contraction activation. The basic idea of the model is that the rate limiting process is calcium movement in the sarcomere due to high concentrations of fast calcium binding sites. This is in a good accordance with the measurements of the calcium diffusion constant by Kushmerick and Podolsky (1969). They found very low value $D = 14\ \mu m^2 \cdot s^{-1}$, which is almost two orders lower than in the free solution. This value is close to the square of parameter $k$ as determined in our experiments.

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