Cytoplasmic Free Concentrations of Ca^{2+} and Mg^{2+} in Skeletal Muscle Fibers at Rest and during Contraction

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Summary: This review summarizes estimates for cytoplasmic-free concentrations of Ca^{2+} ($[\text{Ca}^{2+}]_i$) and Mg^{2+} ($[\text{Mg}^{2+}]_i$) at rest and during contraction of skeletal muscles, from which substantial quantitative information about them has been accumulated. Although the estimates of resting $[\text{Ca}^{2+}]_i$ in the literature widely differ, which is because of the variety of difficulties related to different methodologies used, recent studies suggest that estimates of resting $[\text{Ca}^{2+}]_i$ of approximately 0.05–0.1 μM are likely to be correct. Following action potential propagation, the Ca^{2+} release from the sarcoplasmic reticulum causes a transient rise of $[\text{Ca}^{2+}]_i$ (Ca^{2+} transient). The large peak amplitude and brief time course of the Ca^{2+} transients have been established only recently by studies with low-affinity Ca^{2+} indicators

developed in the past decade. These technical improvements in $[\text{Ca}^{2+}]_i$ measurements have made it possible to study relationships between $[\text{Ca}^{2+}]_i$ and force in intact muscle fibers. In the second part of this review, various estimates of $[\text{Mg}^{2+}]_i$ in the resting muscle are discussed. Relatively recent estimates of the $[\text{Mg}^{2+}]_i$ level appear to be about 1.0 mM. Using the current knowledge of concentrations and reaction properties of intracellular Ca^{2+} - Mg^{2+} binding sites, we constructed a model for dynamic Mg^{2+} movement following Ca^{2+} transients. The model predicts that with a train of action potentials, the sustained rise of $[\text{Ca}^{2+}]_i$ produces an elevation of $[\text{Mg}^{2+}]_i$ of about 200 μM . [Japanese Journal of Physiology, 48, 421–438, 1998]

Key words: skeletal muscle, muscle contraction, cytoplasmic Ca^{2+} , cytoplasmic Mg^{2+} , Ca^{2+} transient.

It is well established that skeletal muscle contraction is regulated by cytoplasmic-free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$). Following action potential propagation along the transverse tubular membrane, Ca^{2+} is released from sarcoplasmic reticulum (SR) to produce a transient rise of $[\text{Ca}^{2+}]_i$ (Ca^{2+} transient) (e.g., Endo [1]). Ca^{2+} binding to regulatory (Ca^{2+} -specific) sites of troponin on thin myofilaments triggers contraction, and the dissociation of Ca^{2+} from the sites leads to relaxation (e.g., Ebashi [2]). Contractile force is a function of peak amplitude and time course of the Ca^{2+} transients. During brief Ca^{2+} transients, contractile force lags behind $[\text{Ca}^{2+}]_i$ because of the delay in steps

between Ca^{2+} binding to troponin and force generation. As a consequence, the peak contractile force is smaller than that expected from the steady-state $[\text{Ca}^{2+}]_i$ -force relation. When the change in $[\text{Ca}^{2+}]_i$ is slow enough to reach equilibrium with contractile force, the steady-state force can be generated. Thus a knowledge of the kinetic and quantitative relationships between Ca^{2+} transients and contraction is important to understand how muscle contraction is regulated by Ca^{2+} . Besides the importance of the Ca^{2+} transients, the level of resting $[\text{Ca}^{2+}]_i$ before activation may also strongly influence muscle function. With the elevation of resting $[\text{Ca}^{2+}]_i$, the Ca^{2+} pump of the SR

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is activated and the Ca^{2+} release channel of the SR may be activated [3, 4] or inactivated [5]. An elevated $[\text{Ca}^{2+}]_i$ also partially saturates cytoplasmic Ca^{2+} binding sites (Table 3), leading to a reduction of intracellular Ca^{2+} buffering power.

On the other hand, the physiological roles of Mg^{2+} , the second or third most abundant intracellular cation, are not yet clear. Cytoplasmic free concentration of Mg^{2+} ($[\text{Mg}^{2+}]_i$) may have profound effects via many different pathways: (1) Ca^{2+} release from the SR (for review, see Endo [1]); (2) the $[\text{Ca}^{2+}]_i$ -force relation of the myofilaments (e.g., Donaldson and Kerrick [6]); (3) the turnover rate of the SR Ca^{2+} pump (for review, Inesi [7]); (4) competition for Ca^{2+} - Mg^{2+} binding sites with Ca^{2+} [8] (Figs. 2 and 3). However, technical difficulties have hampered an accurate monitoring of the level of $[\text{Mg}^{2+}]_i$, especially changes in $[\text{Mg}^{2+}]_i$ during muscle activity.

To define the reasonable range of $[\text{Ca}^{2+}]_i$ and $[\text{Mg}^{2+}]_i$ encountered at rest and during muscle contraction, the quantitative results on these ions reported for skeletal muscle will be summarized. The focus is on frog skeletal muscle fibers, in which $[\text{Ca}^{2+}]_i$ and $[\text{Mg}^{2+}]_i$ have been studied most extensively and also in which the likely composition of the cytoplasm has been specified (for review, Godt and Maughan [9]). The review contains model calculations for Ca^{2+} transient-associated changes in $[\text{Mg}^{2+}]_i$, constructed by using representative data for major cytoplasmic constituents.

Cytoplasmic Free Concentrations of Ca^{2+} and Mg^{2+}

In this article, $[\text{Ca}^{2+}]_i$ and $[\text{Mg}^{2+}]_i$ refer to the spatially averaged free concentrations of these ions in the cytoplasm. In the resting muscle fibers, $[\text{Ca}^{2+}]_i$ and $[\text{Mg}^{2+}]_i$ levels in the sarcomere are thought to be homogeneous. However, when Ca^{2+} release from the terminal cisternae of SR is activated, the concentration gradient of cytoplasmic-free Ca^{2+} is expected to develop. When the SR Ca^{2+} release is then turned off, cytoplasmic Ca^{2+} diffusion causes the gradient to dissipate [10, 11]. During the Ca^{2+} transient, therefore, the local level of $[\text{Ca}^{2+}]_i$ within the sarcomere is higher or lower than $[\text{Ca}^{2+}]_i$, in accordance with the distance from the Ca^{2+} release channels of the SR and the time after initiation of the release. The concentration gradient of Mg^{2+} may also be formed during the Ca^{2+} transient if Mg^{2+} is released in exchange for Ca^{2+} from the local binding sites for both Ca^{2+} and Mg^{2+} . These localized changes in $[\text{Ca}^{2+}]_i$ and $[\text{Mg}^{2+}]_i$ are complex functions of intrasarcomeric geometry and time, and are not addressed in the present article.

Resting $[\text{Ca}^{2+}]_i$

Measurement with Ca^{2+} -selective electrodes

Two varied types of methods have been applied to estimate the resting levels of $[\text{Ca}^{2+}]_i$: ion selective electrodes and optical indicators (Table 1). Ca^{2+} -selective electrodes (Ca-ISEs) are prepared by filling the tip of glass microelectrodes with Ca^{2+} exchange resin. Among the Ca^{2+} exchange resins introduced, the neutral sensor, ETH-1001, has been used most successfully [12] (Table 1). The Ca-ISEs have a well-defined theoretical basis, and the output of the electrodes should be directly related to the $[\text{Ca}^{2+}]_i$ at the tip, ideally, a ~ 30 mV change for a 10-fold change in Ca^{2+} activity. However, at very low Ca^{2+} levels around resting $[\text{Ca}^{2+}]_i$ and in the presence of ions that interfere with the electrodes (the most important being K^+ , Na^+ , and Mg^{2+}), the response of actual electrodes shows much shallower slopes than that expected from Nernstian behavior (for review, see Blinks *et al.* [13]). Thus the small errors in the estimation of the cytoplasmic concentration of interfering ions and in membrane potential subtraction lead to a nonnegligible difference in the estimation of Ca^{2+} concentration. Another potential source of error of the Ca-ISEs is the leakage of Ca^{2+} around the sites of mechanical impalement, which tends to elevate the local $[\text{Ca}^{2+}]_i$. Some estimates obtained with Ca-ISEs listed in Table 1 may be erroneously high as a consequence of local membrane damage, as pointed out by Weingart and Hess [14].

Measurement with optical indicators

Five major classes of optical Ca^{2+} indicators are currently in use [15]: (1) photoproteins, (2) metallochromic dyes, (3) purpurate dyes, (4) tetracarboxylate dyes, and (5) tricarboxylate dyes. Metallochromic dyes, such as arsenazo III and antipyrilazo III, are rarely used for the determination of resting $[\text{Ca}^{2+}]_i$ because of the difficulty in calibrating their absorbance signals, resulting from complex stoichiometries of Ca^{2+} binding and the sensitivity to other ions, such as Mg^{2+} and H^+ [16–19]. Neither are purpurate dyes and tricarboxylate dyes suitable for measurements of low $[\text{Ca}^{2+}]_i$ around the resting level because of their relatively low affinity for Ca^{2+} ; with a dissociation constant (K_D) of ~ 30 μM or higher, the fraction (<0.005) of the indicator molecules expected to be Ca^{2+} -bound in the resting cytoplasm is practically nonmeasurable. [However, these low-affinity dyes are useful for monitoring large and brief Ca^{2+} transients. Tricarboxylate dyes can also be used as Mg^{2+} indicators (see below).]

Among photoproteins, aequorin has been generally employed. Aequorin is weakly luminescent even in

Table 1. Estimates of resting [Ca²⁺]_i in vertebrate skeletal muscles.

Animal species	Method	[Ca ²⁺] _i (μM)	References
Frog	Ca-ISE ^a	0.16	Tsien and Rink [93] (20°C, 1.5 mM [Ca ²⁺] _o)
Frog	Ca-ISE ^a	0.12	López <i>et al.</i> [94] (20–21°C, 1.8 mM [Ca ²⁺] _o)
Frog	Ca-ISE ^a	0.052	Weingart and Hess [14] (20–22°C, 1.8 mM [Ca ²⁺] _o)
Frog	Aequorin ^b	0.027	Moore [22] (20°C, 1.8 mM [Ca ²⁺] _o) [Mg ²⁺] _i was assumed to be 3 mM
Frog	Aequorin	<0.13	Konishi and Kurihara [23] (17–18°C, 1.8 mM [Ca ²⁺] _o) Only the upper limit was estimated ^c
Rat	Ca-ISE ^a	0.145	Ballanyi and Grafe [95] (37°C, 1.5 mM [Ca ²⁺] _o)
Pig	Fura-2 (AM) ^d	0.08	Iaizzo <i>et al.</i> [96] (35°C, 2.5 mM [Ca ²⁺] _o) <i>In vitro</i> calibration
Frog	Aequorin	≤0.038	Blatter and Blinks [24] (18–20°C, 1 mM [Mg ²⁺] _o)
	Ca-ISE ^a	≤0.059	Probably upper limit estimation ^e
Mouse	Indo-1	0.026	Westerblad and Allen [39] (22°C, 1.8 mM [Ca ²⁺] _o) Intracellular calibration by injection of CaCl ₂ and EGTA
Mouse	Fura-2	0.046	Head [36] (probably 22°C, 2.5 mM [Ca ²⁺] _o) Intracellular calibration using a Ca ²⁺ ionophore, combined with other muscle measurements
Frog	Fura red	0.18–0.27 ^f	Kurebayashi <i>et al.</i> [31] (16°C, 1.8 mM [Ca ²⁺] _o) <i>In vitro</i> calibration with intracellular proteins, combined with some intact muscle measurements
Frog	Fluo-3	0.10–0.24 ^f	Harkins <i>et al.</i> [30] (16°C, 1.8 mM [Ca ²⁺] _o) <i>In vitro</i> calibration with intracellular proteins, combined with some intact muscle measurements
Frog	Fura dextran	0.055–0.14 ^f	Konishi and Watanabe [32] (17°C, 1.8 mM [Ca ²⁺] _o) Intracellular calibration in permeabilized fibers
Rat	Fura-2 (AM) ^d	0.039	Carroll <i>et al.</i> [37] (28–30°C, 1 mM [Ca ²⁺] _o) ^g Intracellular calibration with a Ca ²⁺ ionophore, combined with some intact muscle measurements
Toad (<i>Xenopus</i>)	Indo-1	0.052	Westerblad and Allen [40] (22°C, 1.8 mM [Ca ²⁺] _o) Intracellular calibration by injection of CaCl ₂ and EGTA

Examples of estimated values are shown in chronological order. Measurements in cut fibers (e.g., Klein *et al.* [33]) are not included in the table because cut fibers are internally perfused with artificial solutions. ^aCa²⁺ selective electrodes were constructed with the sensor ETH-1001. ^bNative and acetylated aequorin. ^c[Ca²⁺]_i levels were elevated by the application of 0.6 mM caffeine. ^dThe preparation was loaded with fura-2 by incubation with its acetoxymethyl (AM) ester form. ^eMeasurements in fibers slightly depolarized by 12.5 mM extracellular K⁺. ^fThe range covers estimates with two different calibration methods. ^gThe authors found no change in [Ca²⁺]_i either by lowering temperatures to 25°C, from 37°C (for ~1 h), or by changing [Ca²⁺]_o from 1.0 to 2.5 mM.

the virtual absence of Ca²⁺ (Ca²⁺-independent luminescence), but it emits large additional luminescence on Ca²⁺ binding [20]. A calibration of aequorin luminescence in terms of [Ca²⁺]_i has usually been carried out with the method introduced by Allen and Blinks [21]. After measurements of luminescence (*L*) from muscle fibers injected with aequorin, the fiber is lysed by Triton X-100 to expose all aequorin molecules in the fiber to Ca²⁺. By using the time integral of light intensity at full discharge of aequorin, the maximum luminescence that would be emitted at saturating [Ca²⁺]_i (*L*_{max}) can be calculated. Fractional luminescence (*L/L*_{max}), the light intensity from a given amount of aequorin, is then converted to [Ca²⁺]_i by using a calibration curve constructed *in vitro* under appropri-

ate temperature and ionic conditions (pH, [Mg²⁺]). The difficulty in the use of aequorin to determine resting [Ca²⁺]_i is that the fractional light level obtained from resting muscle is very close to the Ca²⁺-independent luminescence [22–24]; even a small error in the *in vitro* calibration curve will largely influence the estimates of [Ca²⁺]_i. Moore [22] reported an estimate of [Ca²⁺]_i of 27 nM under the assumption that [Mg²⁺]_i was 3 mM. However, if his results are reanalyzed with a calibration curve obtained by using the more likely level of 1 mM [Mg²⁺]_i (see below), the fractional luminescence levels of most resting fibers are below Ca²⁺-independent luminescence of the calibration curve, giving “negative” values for [Ca²⁺]_i (see Blatter and Blinks [24]). Blatter and Blinks [24] also

found apparently “negative” $[Ca^{2+}]_i$ with the calibration in the presence of 1 mM $[Mg^{2+}]$. A possible reason for the “negative” estimates for $[Ca^{2+}]_i$ is that myoplasm contains a diffusible macromolecule that interacts with aequorin to reduce Ca^{2+} -independent light emission [24]. Thus until now only upper-limit estimates of resting $[Ca^{2+}]_i$ have been obtained from aequorin under experimental conditions in which $[Ca^{2+}]$ was raised (and L/L_{max} was increased well above the Ca^{2+} -independent level) either by partial depolarization [24] or by the application of caffeine [23] (Table 1).

Fura-2 and indo-1 are the first tetracarboxylate indicators that display spectral shift on binding Ca^{2+} (ratiometric indicators); an analysis of fluorescence intensities at two wavelengths instead of at only a single wavelength, is proposed to alleviate potential artifacts because of changes in dye concentration and cell movement [25]. The high Ca^{2+} -affinity of these indicators (and of many other members of the tetracarboxylate family) is also advantageous for monitoring $[Ca^{2+}]_i$ around resting levels. However, among a number of measurements of $[Ca^{2+}]_i$ made with fluorescent tetracarboxylate dyes, only a few studies have provided useful information on the precise $[Ca^{2+}]_i$ values in muscle fibers (Table 1). This is primarily because of difficulties related to the calibration of indicator signals in terms of $[Ca^{2+}]_i$. In general, Ca^{2+} indicator molecules are heavily bound to cellular constituents (possibly proteins) within the cytoplasm [26]. This binding alters the spectral and binding properties of the indicators; concerning tetracarboxylates, *in vitro* studies show that the binding to soluble proteins changes both fluorescence and Ca^{2+} binding of fura-2 [27, 28], indo-1 [29], fluo-3 [30], and fura-red [31]. In frog skeletal muscle fibers, the fluorescence spectra of fura-2 is shifted to longer wavelengths [32], and as a consequence a simple comparison of the fluorescence ratio of fura-2 in the cytoplasm and in salt solutions lacking proteins indicates “negative” $[Ca^{2+}]_i$ [33, 34]. The influence of cytoplasmic constituents cannot be avoided even by the conjugation of fura-2 to high molecular weight dextran (fura dextran ~ 10 kD); fura dextran injected into cytoplasm exhibited a red shift in the fluorescence excitation spectrum and about a twofold increase of K_D for Ca^{2+} [32]. Thus the simple assumption that indicators behave the same in the intracellular environment as they do in salt solutions is probably not valid, requiring calibration in the “intracellular” environment.

Methods that have been proposed include (1) a permeabilization of the cell membrane by Ca^{2+} ionophores [35–37] or β -escin [32, 38] to equilibrate

$[Ca^{2+}]_i$ with extracellular $[Ca^{2+}]$; (2) the injection of a high concentration of $CaCl_2$, EGTA, or a mixture of the two into muscle fibers [39, 40]; (3) a combination of the measurement of optical signals from intact muscle fibers and *in vitro* solution containing cytoplasmic protein [30, 31]. It is not reasonable, however, to place strong reliance on any single calibration method at this point [41, 42]. If the indicators are introduced into the cells by incubation with the acetoxymethyl ester (AM) derivatives, an additional problem could arise from possible compartmentalization; membrane-permeant AM ester of the indicator can enter the intracellular organelles and, as a result of hydrolysis, may be trapped within them. With this compartmentalization, a significant fraction of the indicator signals could reflect $[Ca^{2+}]$ in the organelles, such as in SR and mitochondria, introducing errors in measurement of $[Ca^{2+}]_i$.

Likely range of resting $[Ca^{2+}]_i$

Technical difficulties inherent in each method discussed in the previous section probably contribute to the 10-fold difference for estimates of $[Ca^{2+}]_i$ listed in Table 1 (0.03–0.3 μM). Harkins *et al.* [30], using fluo-3, suggest a 0.10–0.24 μM range for resting $[Ca^{2+}]_i$ of frog muscle fibers. Their range covers two different estimates for $[Ca^{2+}]_i$ from two values of the apparent K_D of fluo-3 for Ca^{2+} ; the low-end estimate (0.10 μM) is based on a K_D value obtained in calibration solutions containing aldolase, a major cytoplasmic protein, and the high-end estimate (0.24 μM) is based on kinetic fits of fluo-3 fluorescence signals with Ca^{2+} transients in intact muscle fibers. However, the kinetic fits originally introduced by Baylor *et al.* [43] and Klein *et al.* [33] assume uniform distribution of Ca^{2+} in the cytoplasm, which is unlikely to occur during brief Ca^{2+} transients [10]. Baylor and Hollingworth [11] recently used a multicompartment model for the kinetic analysis and reported that the single compartment method would introduce an overestimation of K_D (therefore an overestimation of $[Ca^{2+}]_i$) because of local saturation of the indicator. A reanalysis of the fluo-3 data [30] lowered the estimates for $[Ca^{2+}]_i$ to 0.14 μM [11], from 0.24 μM . Thus the data of Harkins *et al.* [30] imply a smaller range of values of $[Ca^{2+}]_i$ (0.10–0.14 μM). It is likely that other values reported from the kinetic fits with a single compartment approach (0.18 μM , Kurebayashi *et al.* [31]; 0.14 μM , Konishi and Watanabe [32]) should also be adjusted downward.

Blatter and Blinks [24] found that both aequorin and Ca-ISEs often showed inconceivably low $[Ca^{2+}]_i$ levels even after $[Ca^{2+}]_i$ was raised by partial depolar-

ization. For example, the response of the Ca-ISE in each fiber gave widely variable values for [Ca²⁺]_i from a largely negative value to ~1.8 μM (their table 1), which forced them to use the average value of the electrode response to estimate the average [Ca²⁺]_i of 10 muscle fibers. Although their experiments were carefully carried out and the average [Ca²⁺]_i estimates from aequorin and the Ca-ISEs agreed fairly well, this technical limitation and the existence of a macromolecule that influences aequorin light emission (see the previous section) affect the accuracy of their estimation.

These considerations help in reconciling the values reported with different techniques (Table 1). For frog muscle fibers, the general assumption of resting [Ca²⁺]_i within or close to the 0.05 to 0.1 μM range seems to be reasonable. The levels of [Ca²⁺]_i may be somewhat lower in mammalian muscle fibers, but more studies employing different methodologies must be undertaken. It should be noted that no promising method has been established and that all values in Table 1 may be the subject of future reevaluation.

Changes in [Ca²⁺]_i Following Action Potential Propagation

Time course

To track brief Ca²⁺ transients following depolarization, optical indicators have been exclusively used; Ca-ISEs suffer a serious disadvantage because of their slow response and the difficulty in maintaining stable cell membrane impalement during cell motion. Although numerous studies have used measurements of Ca²⁺-related optical signals in skeletal muscle fibers, the reported Ca²⁺ transients using a variety of indicators differ significantly in their time course and in their peak amplitude. For example, in frog skeletal muscle at 16–18°C, the time to peak of the optical signals varies from 5 to 20 ms after single-action potential stimulation: 15–18 ms with aequorin [23, 44]; 12–13 ms with arsenazo III [45]; 6–8 ms with antipyrilazo III [46, 47]; ~5 ms with tetramethylmurexide [48, 49], purpurate diacetic acid [49, 50], and dimethylpurpurate diacetic acid [50]; 5–6 ms with fura-2 [51]; 14–18 ms with fura-2 [32, 47]; ~15 ms with fluo-3 [30]; and ~20 ms with fura red [31] (for many other tetracarboxylate dyes and tricarboxylate dyes, see Zhao *et al.* [26]). These large differences in the time course of the indicator signals are likely due to the different reaction kinetics of each indicator for Ca²⁺ and to the contribution of the effects of local Ca²⁺ gradients (cf. Baylor and Hollingworth [11]).

If indicator molecules in the Ca²⁺-free form (D) react with Ca²⁺ to form a complex (CaD) with 1:1

stoichiometry, the rate of formation of the complex (d[CaD]/dt) is expressed by this kinetic equation:

$$d[\text{CaD}]/dt = k_{+1}[\text{Ca}^{2+}][\text{D}] - k_{-1}[\text{CaD}], \quad (1)$$

where k_{+1} is the association rate constant and k_{-1} is the dissociation rate constant. The dissociation equilibrium constant (K_D) is defined as k_{-1}/k_{+1} . For a step change in [Ca²⁺] to a new level of [Ca²⁺] n times larger than K_D , the Ca²⁺-indicator complex formation (and therefore the optical signal) follows the change in [Ca²⁺] with a first-order rate constant of $(n+1) \cdot k_{-1}$ [52]. Therefore optical signals from indicators with a larger k_{-1} can track Ca²⁺ transients with smaller kinetic delay; the tendency for low-affinity Ca²⁺ indicators to display faster optical signals is expected if the greater K_D value of the low-affinity indicator is due to a greater k_{-1} with a similar k_{+1} . In practice, signals from high-affinity indicators generally require kinetic correction to more accurately follow the time course of Ca²⁺ transients.

Chandler and co-workers first demonstrated the usefulness of low-affinity indicators. After the initial observation with tetramethylmurexide (TMX) [48], new purpurate indicators, purpurate diacetic acid (PDAA), and dimethylpurpurate diacetic acid (DMPDAA) were introduced [53]. PDAA and DMPDAA both have a low affinity for Ca²⁺ (K_D 0.8–1.0 mM range), and their absorbance signals appear to track Ca²⁺ transients without kinetic delay when applied to frog cut muscle fibers [50]. The time course of the Ca²⁺ transients from these indicators was found to be very brief: time to peak ~5 ms; half-width 7–9 ms (16°C). Ca²⁺ transients with a very similar time course were detected in intact frog muscle fibers injected with PDAA [49]. Fluorescence signals of fura-2, a low-affinity tricarboxylate dye (K_D ~50 μM for Ca²⁺), also had a similar temporal waveform. Fura-2's fluorescence signal may lag behind the PDAA's absorbance signal by only ~1 ms [51]. (Fura-2 is also called mag-fura-2, because it was originally designed as an Mg²⁺ indicator [54].) In general, low-affinity indicators of the tricarboxylate family ($K_D \geq 30$ μM) appear to track the Ca²⁺ transient with very minor delay, whereas high-affinity indicators of the tetracarboxylate family ($K_D \leq 0.6$ μM) significantly lag behind the Ca²⁺ transient. Thus the slow time course of the optical signals of many indicators (especially those with high Ca²⁺-affinity) reflects a significant delay introduced by small k_{-1} .

Peak amplitude

Besides to the difference in the time course, peak amplitude of the Ca²⁺ transients calibrated from each

indicator is different. Even among indicators with apparently minor kinetic delay, the calibrated peak amplitude spans a range from 2 to 3 μM with antipyrilazo III [46, 47] to 21 μM with PDAA and DMPDAA [50]. This difference of almost an order of magnitude is probably due to the use of K_D values estimated *in vitro* for conversion of the indicators' optical signals to $[\text{Ca}^{2+}]_i$; the "intracellular" calibration technique has not been carried out for low-affinity indicators for Ca^{2+} , probably because of difficulties to establish $[\text{Ca}^{2+}]_i$ levels high enough to saturate indicator molecules without destroying the cell structure.

As described in the previous section, indicator molecules appear to readily bind to cell constituents when introduced into cytoplasm. Consequently, any estimate of $[\text{Ca}^{2+}]_i$ depends on whether these bound indicator molecules are able to react with Ca^{2+} in the same manner as indicator molecules react in calibration solutions. With the purpurate dyes (PDAA and DMPDAA), the percentage of indicator molecules bound to cytoplasmic constituents was found to be relatively small (19 to 27% in cut fibers [50] and 24 to 43% in intact fibers [49]) in comparison with 42–51% found for fura-2 [51] and the major bound fraction detected with antipyrilazo III ($\sim 75\%$ [48, 55]), as well as many other indicators (~ 60 to 90% [26, 30, 31, 47]). Since the indicator with a smaller bound fraction is likely to yield a more reliable value for $[\text{Ca}^{2+}]_i$, it is likely that the large peak amplitude reported by purpurate indicators is more reliable than that calibrated from the heavily bound ones. In the case of fura-2, which appears to bind to cytoplasmic constituents less than many other dyes do (but more than purpurate dyes), the calibrated peak amplitude was found to be about half that obtained with PDAA. Under the assumption that PDAA correctly indicates the peak amplitude of the Ca^{2+} transient, the K_D of fura-2 for Ca^{2+} may be increased by a factor of two as a consequence of binding to cellular constituents [49, 51].

In intact frog muscle fibers, the peak amplitude of Ca^{2+} transients was initially estimated to be about 10 μM from PDAA [49], which was about half that estimated in cut muscle fibers [50]. In a subsequent study carried out in intact muscle fibers with fura-2, however, calibration with the twofold correction for fura-2's K_D for Ca^{2+} (see the previous paragraph) gave a larger average value for peak Ca^{2+} transients, 16.5 μM [26]. The reason for this variation is unknown, but it may reflect a biological variation in the amplitude of Ca^{2+} transients among different groups of frogs. Therefore the reasonable range for the peak amplitude of Ca^{2+} transients appears to be 10 to 20 μM for frog skeletal muscle fibers (either intact or cut fibers) stim-

ulated by an action potential.

It is interesting that Ca^{2+} transients appear to be as brief and as large in mammalian skeletal muscle fibers as they are in frog fibers. In fast twitch fibers from extensor digitorum longus muscle of mice, the amplitude and the time to peak of the Ca^{2+} transients calibrated from fura-2 (in a way similar to that for frog fibers) were, respectively, 17.8 μM and 4.4 ms for a single twitch at 16°C [56]. At 28°C , a higher peak value (on average 22.1 μM) and a much shorter time to peak (on average 2.0 ms) were found [56].

Late sustained phase

After the peak, Ca^{2+} transient rapidly returns close to the prestimulus baseline within ~ 100 ms, but it remains slightly elevated above the baseline for a long time. A study with fura-2, a high-affinity tetracarboxylate dye, indicates that 100–200 ms after a single-action potential, the quasi-steady-state elevation was 1 to 2% of the peak amplitude of the Ca^{2+} transient [47]. Moreover, this maintained level, studied with fluorescence ratio signals of fura-2, does not recover to the baseline at least for 30 s following a twitch, and even for 90 s after 1-s tetanus in frog muscle fibers [34]; these small, slow changes of $[\text{Ca}^{2+}]_i$ at such a later stage are difficult to study and have not been quantitatively characterized.

Steady-State Relation between $[\text{Ca}^{2+}]_i$ and Force

The steady-state relation between $[\text{Ca}^{2+}]_i$ and force has been studied primarily with skinned fibers in which the cell membrane is removed to easily establish the known levels of $[\text{Ca}^{2+}]_i$ in the myofibrillar space [57, 58]. In spite of the usefulness of skinned fibers, it is desirable to obtain a quantitative relation between $[\text{Ca}^{2+}]_i$ and force in intact muscle fibers because the removal of the cell membrane should produce a large alteration in the environment surrounding the myofibrils and may influence the $[\text{Ca}^{2+}]_i$ -force relation.

In intact muscle fibers stimulated by either a single-action potential or a train of action potentials, force generation follows a rapid rise and fall of $[\text{Ca}^{2+}]_i$ with a significant delay, forming a nonequilibrium relation between $[\text{Ca}^{2+}]_i$ and force. However, under experimental conditions that significantly slow the change in $[\text{Ca}^{2+}]_i$ to reach equilibrium with force generation, simultaneous measurements of $[\text{Ca}^{2+}]_i$ and force could provide information on the steady-state relation between $[\text{Ca}^{2+}]_i$ and force. This strategy has long been proposed, but reliable experiments have been hampered by the technical difficulties in quantification of

[Ca²⁺]_i as discussed above. Therefore the precise relation by which [Ca²⁺]_i regulates force is only beginning to be elucidated.

In a series of experiments with mouse skeletal muscle fibers (sarcomere length $\sim 2.3 \mu\text{m}$, 22°C), Westerblad and Allen [39, 59] calibrated indo-1 fluorescence signals by intracellular injection of a large amount of CaCl₂ and EGTA. From the levels of [Ca²⁺]_i and force during the plateau of tetani, they described the [Ca²⁺]_i-force relation by a Hill curve with an average Hill coefficient (a measure of the steepness) of 5.1 to 5.5 and an average Ca₅₀ (the [Ca²⁺]_i giving 50% maximum force) of $\sim 0.4 \mu\text{M}$. By use of an analogous method, a similar relation was reported for toad (*Xenopus*) fast twitch muscle fibers; the average values for the Hill coefficient and Ca₅₀ were, respectively, 4.1 and $0.65 \mu\text{M}$ [60]. Konishi and Watanabe [61] measured [Ca²⁺]_i with fura dextran in frog skeletal muscle fibers and plotted [Ca²⁺]_i versus force during high K⁺ contractures (Fig. 1) and during the slow relaxation phase after tetani in the presence of an inhibitor of the SR Ca²⁺ pump. The [Ca²⁺]_i-force data obtained from the two types of measurements agreed fairly well, consistent with an average Hill coefficient of 3.2 to 3.9 and an average Ca₅₀ of $1.5\text{--}1.7 \mu\text{M}$ (sarcomere length 2.6 to $2.8 \mu\text{m}$, 16–18°C). The two- to threefold difference in Ca₅₀ values estimated in toad muscle [60] and frog muscle [61] may reflect calibration errors in one (or both) of these studies, but it may also reflect a true difference in [Ca²⁺]_i between toad and frog muscles. Maximal [Ca²⁺]_i level at the plateau phase of tetani was $\sim 2 \mu\text{M}$ in Westerblad *et al.* [60], whereas it was $\sim 10 \mu\text{M}$ in Konishi and Watanabe [61].

Morgan *et al.* [62] and Claflin *et al.* [63] analyzed the relationship between a slow decline of [Ca²⁺]_i and a relaxation of force following twitches in frog skeletal muscle fibers with an inhibited SR Ca²⁺ pump (sarcomere length 2.2 or $2.9 \mu\text{m}$, 3°C). Although Ca₅₀ value of their [Ca²⁺]_i-force relation is uncertain because of the use of K_D from *in vitro* measurements, a Hill coefficient could be estimated. The values for Hill coefficients estimated in their studies are surprisingly large; on average, 11 and 15 at sarcomere lengths of 2.9 and $2.2 \mu\text{m}$, respectively. The reason for the large difference in Hill coefficients of 3–4 [61] and 11 [63] estimated at a similar sarcomere length in the same frog species is unknown, although the differences in experimental temperature may in part account for the discrepancy (e.g., Godt and Lindley [64]). Possibly, the force measurement in one (or both) of these studies may have been seriously influenced by the shortening and lengthening of segments within the fibers.

Fig. 1. [Ca²⁺]_i (in pCa units) versus force (relative to peak tetanic force) plot in a frog fast muscle fiber (17°C, sarcomere length $2.8 \mu\text{m}$). Symbols represent peak [Ca²⁺]_i and peak force during high K⁺ contractures (20 K, 20 mM K⁺; 30 K, 30 mM K⁺) or tetanus. A solid line is a Hill curve least squares fitted to the symbols with the best-fitted parameters indicated in the panel. The dotted lines represent instantaneous [Ca²⁺]_i versus force plots during K⁺ contracture (30 mM K⁺) and the plateau phase of tetanus. Arrows indicate the direction of the change as a function of time (reproduced from Konishi and Watanabe [61], by copyright permission of the Rockefeller University Press).

Clearly, further improvements for measurements of both [Ca²⁺]_i and force (e.g., sarcomere clamp) are necessary to resolve this important uncertainty about the steady-state relation between [Ca²⁺]_i and force in intact muscle fibers.

Resting [Mg²⁺]_i

Three types of methods have been mostly employed to measure resting [Mg²⁺]_i: nuclear magnetic resonance spectroscopy (NMR), Mg²⁺-selective electrodes (Mg-ISEs), and optical indicators (Table 2).

Measurement with NMR

Most [Mg²⁺]_i measurements with NMR rely on a chemical shift difference between α - and β -ATP resonances of ³¹P-NMR, which uses ATP as an intrinsic Mg²⁺ indicator, but some exogenous Mg²⁺ indicators for NMR have also been introduced (for review, see Gupta *et al.* [65] and London [66]). One advantage for the use of ATP as an NMR indicator is its natural existence in high concentrations in a broad spectrum of cell types; information on cellular Mg²⁺ level can be obtained without any mechanical perturbation to the cells, such as cell membrane impalement with the electrodes. A limitation of ATP as an Mg²⁺ indicator

Table 2. Estimates of resting $[Mg^{2+}]_i$ in vertebrate skeletal muscles.

Animal species	Method	$[Mg^{2+}]_i$ (mM)	References
Frog	^{31}P -NMR	4.4	Cohen and Burt [97] (4°C, 0 mM $[Mg^{2+}]_o$) Their method of analysis has been criticized [65]
Frog	^{31}P -NMR	0.6	Gupta and Moore [70] (25°C ^a , 0–1.6 mM $[Mg^{2+}]_o$) Similar $[Mg^{2+}]_i$ at 0 mM and 1.6 mM $[Mg^{2+}]_o$
Frog	Mg-ISE	3.3	Hess <i>et al.</i> [72] (22–24°C, 0.5 mM $[Mg^{2+}]_o$) Sensor ETH-1117 ^b
Frog	Arsenazo III	0.5–1.2 ^c	Baylor <i>et al.</i> [18] (16°C, 0 mM $[Mg^{2+}]_o$)
	Dichlorophosphonazo III	0.2–0.3 ^c	<i>In vitro</i> calibration
	Arsenazo I	3–6 ^c	
Frog	X-ray microanalysis	0.2	Maughan [98] (20°C, 0 mM $[Mg^{2+}]_o$) Calculated from total Mg concentration
Frog	Mg-ISE	3.8	López <i>et al.</i> [73] (22°C, 0 mM $[Mg^{2+}]_o$) Sensor ETH-1117 ^b
Frog	Mg-ISE	0.8	Alvarez-Leefmans <i>et al.</i> [99] (22–24°C, 0 mM $[Mg^{2+}]_o$) ^d Sensor ETH-1117 with correction for $[K^+]^b$
Cat	^{31}P -NMR	1.5	Kushmerick <i>et al.</i> [71] (24°C)
Frog	Mg-ISE	0.93	Blatter [100] (18–20°C, 1 mM $[Mg^{2+}]_o$) Sensor ETH-5214 ^e
Rat	Mg-ISE	0.47	MacDermott [101] (30°C, 1.2 mM $[Mg^{2+}]_o$) Sensor ETH-1117 with correction for $[K^+]^b$
Mouse	Furaptra	0.78	Westerblad and Allen [74] (22°C, 0.5 mM $[Mg^{2+}]_o$) Intracellular calibration by injection of $MgCl_2$ and EDTA
Toad (<i>Xenopus</i>)	Furaptra	1.7	Westerblad and Allen [78] (22°C, 0 mM $[Mg^{2+}]_o$) Intracellular calibration by injection of $MgCl_2$ and EDTA
Rat	Furaptra (AM) ^f	0.56	Ng <i>et al.</i> [102] (37°C, 0.8 mM $[Mg^{2+}]_o$) <i>In vitro</i> calibration
Frog	Furaptra	0.54–1.1 ^g	Konishi <i>et al.</i> [79] (17°C, 0 mM $[Mg^{2+}]_o$) <i>In vitro</i> calibration with correction for intracellular K_D
Mouse	Mag-indo-1	1.53	Csernoch <i>et al.</i> [77] (1 mM $[Mg^{2+}]_o$) Intracellular calibration in permeabilized fibers

Examples of estimated values are shown in chronological order. Measurements in human subjects (e.g., Buchli *et al.* [103], Ryschon *et al.* [104], and Irish *et al.* [105]) are not included in the table. ^a The authors also reported similar values of $[Mg^{2+}]_i$ at 35°C. ^b The sensor ETH-1117 has the problem of interference with Na^+ and K^+ . ^c The range is from two values estimated for cytoplasmic pH 7.1 and 6.9. ^d The authors also reported a higher average value for $[Mg^{2+}]_i$, 1.69 mM, at 0.5 mM $[Mg^{2+}]_o$. ^e The sensor ETH-5214 has much better selectivity for Mg^{2+} over Na^+ and K^+ compared with ETH-1117. ^f The preparation was loaded with furaptra by incubation with the acetoxymethyl ester form of the indicator. ^g The range includes estimates based on two different assumptions for cytoplasmic K_D (see text for details).

is that ATP also binds H^+ , and this proton binding changes the chemical shifts of ATP and MgATP, requiring careful correction for the pH effect on the basis of accurate knowledge on the cytoplasmic pH (e.g., Nakayama *et al.* [67]). Another important factor is the choice of K_D value of Mg^{2+} binding to ATP. Because the K_D measurement of intracellular ATP has not been made, the apparent K_D value estimated in solutions in the presence of appropriate concentrations of K^+ and H^+ has been assumed. However, a significant scatter is seen for estimates of the apparent K_D of Mg^{2+} : ATP reaction between 45 μM at 0.15 M $[K^+]$, pH 7.2, 25°C (given by Gupta *et al.* [68]) and 148 μM at 0.15 M $[K^+]$, pH 7.1, 22°C (calculated from Table 3 of Fabiato [69]). This uncertainty should not be over-

looked because the calculated $[Mg^{2+}]_i$ is directly proportional to the assumed K_D . Therefore a sizable part of the different estimates of $[Mg^{2+}]_i$ in the literature, e.g., 0.6 mM by Gupta and Moore [70] and 1.5 mM by Kushmerick *et al.* [71] in Table 2, could be attributed to the difference in assumed values for K_D : 45 μM in Gupta and Moore [70] and 78 μM in Kushmerick *et al.* [71].

Measurement with Mg^{2+} -selective electrodes

Mg-ISEs share a very similar theoretical basis and methodological basis with Ca-ISEs. Therefore many of the advantages and disadvantages for the use of Mg-ISEs are common with those of Ca-ISEs described above and are thus not discussed in detail

here. Among the $[Mg^{2+}]_i$ values estimated from the Mg-ISEs in Table 2, the 3–4 mM range reported by earlier studies [72, 73] is thought to be erroneously high because the Mg²⁺ exchange resin ETH-1117 used in those studies has the problem of significant interference with Na⁺ and K⁺. The new Mg²⁺ exchange resin, ETH-5214, which has a greatly improved selectivity for Mg²⁺ over Na⁺ and K⁺, has shown lower estimates for $[Mg^{2+}]_i$ (Table 2).

Measurement with optical indicators

Baylor *et al.* [18] attempted to measure $[Mg^{2+}]_i$ in resting skeletal muscle fibers of frogs with three different metallochromic dyes but found a quite different estimate with each dye (Table 2). The most likely reason for such differences is that once injected, these dyes bind to cellular constituents, and this binding alters dye properties, as suggested by Baylor *et al.* [18] and discussed above for Ca²⁺ indicators.

The relatively recent development of fura-2 [54] and other fluorescent Mg²⁺ indicators of the tricarboxylate family have facilitated measurements of $[Mg^{2+}]_i$ in many types of cells. These dyes, in spite of the markedly reduced affinity for Ca²⁺, still have an affinity about two orders of magnitude higher for Ca²⁺ than for Mg²⁺ and can be used as low-affinity Ca²⁺ indicators during brief and large Ca²⁺ transients (see above). However, at the likely level of resting $[Ca^{2+}]_i$ ~0.1 μ M (see above), these indicators (K_D for Ca²⁺ ~30 to 50 μ M) are expected to primarily bind Mg²⁺. Therefore the tricarboxylate fluorescent indicators act as Mg²⁺ indicators in the resting muscle fibers and as Ca²⁺ indicators in stimulated fibers.

The calibration of optical signals in terms of $[Mg^{2+}]_i$ also suffers the same difficulties as described above for Ca²⁺ calibration; cytoplasmic binding of the indicator to cellular constituents alters indicator properties. Permeabilization of cell membrane by divalent cation ionophores (ionomycin or Br-A23187) used for Ca²⁺ calibration has the problem that an equilibration of $[Mg^{2+}]_i$ with extracellular Mg²⁺ takes a long time [54, 74]. This is probably due to the low Mg²⁺ transport rate of the ionophores and the requirement for a large amount of Mg²⁺ to significantly change $[Mg^{2+}]_i$. Although it is very difficult to preserve cell structure for many hours of ionophore treatment without gross disruption, calibration parameters for intracellular indicators have been reported for mag-indo-1 in rat ventricular myocytes [75] and in smooth muscle strips of guinea pig tenia cecum [76]. A new calibration method in permeabilized fibers, mag-indo-1 injection into saponin-treated silicone-embedded fibers, has been recently proposed [77]. Westerblad and Allen in-

jected a large volume of MgCl₂/EDTA solution into fura-2-injected skeletal muscle fibers of mice [74] and toads [78], as analogously applied for Ca²⁺ calibration of indo-1 [39, 40]. Again it is important to note that each approach suffers from different limitations and that total reliance on any single method does not seem to be justified now.

Likely range of resting $[Mg^{2+}]_i$

Among the listed values in Table 2, relatively recent estimates for resting $[Mg^{2+}]_i$ in skeletal muscle fibers of frogs and other species seem to be scattered around 1 mM. Konishi *et al.* [79] used *in vitro* calibration for fura-2 signals in frog skeletal muscle, but included assumptions for a possible increase in K_D in the cytoplasm. When the value of K_D for Mg²⁺ obtained *in vitro* was unchanged in the cytoplasm, the calibrated $[Mg^{2+}]_i$ averaged 0.54 mM (lower limit of the range in Table 2). However, if the indicator reacts with Mg²⁺ with a K_D in the cytoplasm twofold larger, as suggested for the Ca²⁺-fura-2 reaction (see above), the calculated $[Mg^{2+}]_i$ would average 1.1 mM (upper limit of the range in Table 2). It may be interesting to reanalyze their results by using the recently estimated values of R_{min} (fluorescence ratio of Mg²⁺-free fura-2), R_{max} (fluorescence ratio of Mg²⁺-bound fura-2), and K_D in smooth muscle cells [76], in spite of the possible species and tissue differences in the calibration parameters. Under the assumption of $R_{min}=0.986$, $R_{max}=0.199$, and the 46% increase in K_D in the cytoplasm of frog muscles, an average $[Mg^{2+}]_i$ of 0.61 mM is calculated from 12 fibers in Table 1 of Konishi *et al.* [79]. The theoretical consideration of the electroneutrality and osmolality of major ionic species in the cytosol is also consistent with $[Mg^{2+}]_i$ of 0.8 mM [9]. Thus in spite of a significant variation in the estimated values, resting $[Mg^{2+}]_i$ around 1 mM is likely to apply in skeletal muscles. The variation that still remains could be attributed to the technical difficulties described above, but it could also reflect a true difference in $[Mg^{2+}]_i$ in different animal species or experimental conditions.

Changes in $[Mg^{2+}]_i$ Following Action Potential Propagation

Experimental results

In frog skeletal muscle fibers stimulated by an action potential, the Ca²⁺-related absorbance change of antipyrylazo III, a metallochromic indicator, occurs only briefly and returns very close to the baseline within 100–200 ms. However, antipyrylazo III also exhibits a slow and sustained change in absorbance with a wavelength dependence that is consistent with the

increase in either $[Mg^{2+}]$ or pH [52, 80]. Irving *et al.* [81] found in cut muscle fibers that the rate of development of the slow component was consistent with the rate of Mg^{2+} dissociation from parvalbumin, but it was very different from the time course of cytoplasmic pH change observed with two pH indicators. Jacquemond and Schneider [82] reported that intracellular perfusion of cut fibers with a solution of very low $[Mg^{2+}]$ (25 μ M) abolished the slow component, but the internal solution with a markedly increased pH buffering capacity did not. These results strongly suggest that the antipyrylazo III slow component primarily reflects an increase in $[Mg^{2+}]_i$. The origin of the observed increase in $[Mg^{2+}]_i$ is most likely attributed to Mg^{2+} leaving parvalbumin in exchange for Ca^{2+} binding [8, 45–48, 81, 82].

Tricarboxylate dyes have not yet been used for monitoring changes in $[Mg^{2+}]_i$ following the Ca^{2+} transients. These dyes undergo very similar spectral changes of fluorescence (and absorbance) for Ca^{2+} and Mg^{2+} [54], making correction for the dominant Ca^{2+} -related component very difficult. From indirect evidence, Konishi *et al.* [51] estimated that about 20 to 40% of the tail of the fura-2 fluorescence signal at later times after stimulation was related to sustained elevation of $[Ca^{2+}]_i$, and the rest was due to the slow increase in $[Mg^{2+}]_i$.

General basics of computer simulation

Because Mg^{2+} movement during muscle contraction has not been well understood in intact muscle fibers, computer simulation was undertaken to estimate the Ca^{2+} transient-associated change in $[Mg^{2+}]_i$ (Mg^{2+} transient) and to evaluate the relative contribution of intracellular binding sites to the Mg^{2+} transients. The model of frog skeletal muscle fibers assumes that any change in $[Mg^{2+}]_i$ is due to alteration in the Mg^{2+} binding of cytoplasmic sites that bind both Ca^{2+} and Mg^{2+} (Ca^{2+} - Mg^{2+} binding sites); i.e., Mg^{2+} is released from the sites in exchange with Ca^{2+} without any change in total Mg^{2+} content in the cytoplasmic pool. This assumption seems to be justified because (1) Mg^{2+} influx through the cell membrane can be neglected, because the Ca^{2+} transients used for the calculation were obtained in Mg^{2+} -free Ringer's solution; (2) Mg^{2+} extrusion, if it exists, is probably too slow to significantly occur in the time scale of the calculation (e.g., Konishi *et al.* [79]); (3) Mitochondria occupy only 1% of the fiber volume [83], and their Mg^{2+} content is unchanged after 1.2 s tetanus [84]. The model, however, may somewhat overestimate the increase in $[Mg^{2+}]_i$ because the Mg content of the SR is slightly increased after 1.2 s tetanus [84].

The basic calculation procedure is the same as the one employed by others [8, 10, 45, 46, 48, 85], but additional consideration is made for several Ca^{2+} - Mg^{2+} binding sites. Brief descriptions of these sites included in the model are as follows:

Troponin: Each troponin molecule on the thin filaments has two low-affinity binding sites and two high-affinity binding sites for Ca^{2+} . Because the low-affinity ("regulatory") sites are thought to be Ca^{2+} specific and are unlikely to release Mg^{2+} on Ca^{2+} binding, Ca^{2+} binding to the troponin low-affinity sites is not included in the model. Only the high-affinity sites that bind both Ca^{2+} and Mg^{2+} are considered.

Myosin: Both heads of myosin have a Ca^{2+} - Mg^{2+} binding site. These sites are mostly occupied with Mg^{2+} in the resting state and are expected to release Mg^{2+} at a very slow rate following the Ca^{2+} transient.

Parvalbumin: A Ca^{2+} and Mg^{2+} binding protein found in brain [86] and fast-twitch skeletal muscle [87] has two binding sites per molecule. Frog skeletal muscle is one of the richest sources of parvalbumins, which is thought to play a role in muscle relaxation [88]. The reported K_D values for Ca^{2+} and Mg^{2+} binding are quite different between parvalbumins from frog muscle [89] and carp muscle [90]. The present simulation uses values for frog muscle, and many other studies have used values for carp muscle (see below). Consequently, the calculated Ca^{2+} and Mg^{2+} binding of parvalbumin in the present model are quite different from those reported by earlier simulation studies [8, 10, 45, 46, 48, 85]. It is unknown whether the difference in the reported K_D values reflects genuine species differences or technical difficulties in the measurement of K_D .

ATP: The apparent K_D values of ATP for Mg^{2+} and Ca^{2+} in the literature show some ranges, as mentioned above. The present calculation uses values given by Fabiato and Fabiato [91], which are somewhat higher than those given by Godt and Lindley [64] (see below).

Creatine phosphate: Even though the affinity of creatine phosphate for Mg^{2+} is very low, the large quantity contained in skeletal muscle makes its contribution to Mg^{2+} movement significant.

Inorganic phosphate (P_i): The apparent K_D 's of inorganic phosphate (mixture of $H_2PO_4^-$ and HPO_4^{2-}) are not very different from those of creatine phosphate. Inorganic phosphate is likely to play a minor role relative to creatine phosphate because of its much smaller abundance in skeletal muscle fibers.

More detailed information on these sites is listed in Table 3. Total $[Mg^{2+}]$ is calculated with the values in Table 3 to be 9.4 mM at 1.0 mM $[Mg^{2+}]_i$. If Mg^{2+}

Table 3. Ca²⁺-Mg²⁺ binding sites.

(1) Species	(2) [Total site] (mM)	(3) Metal	(4) K_D (μ M)	(5) k_{+1} (M ⁻¹ s ⁻¹)	(6) k_{-1} (s ⁻¹)	(7) [Metal site] (fraction) (mM)
Troponin	0.12×2 ^{*a}	Ca	0.002 ^b	3.00×10 ⁸	0.6 ^c	0.119 (0.495)
		Mg	20 ^b	1.0 ×10 ⁵ †	2	0.119 (0.495)
Myosin	0.2×2 ^{*d}	Ca	0.033 ^e	1.38×10 ⁷	0.46 ^f	0.0043 (0.011)
		Mg	3.6 ^e	1.57×10 ⁴	0.057 ^f	0.394 (0.986)
Parvalbumin	0.76×2 ^{*g}	Ca	0.13 ^h	1.10×10 ⁷ ‡	1.4	0.449 (0.296)
		Mg	1,150 ^h	0.55×10 ⁴ ‡	6.3	0.499 (0.328)
ATP	6.2 ⁱ	Ca	300 ^{k,l}	1.0 ×10 ⁸ †	30,000	— (<0.0001)
		Mg	150 ^l	1.00×10 ⁶ m	150	5.39 (0.870)
Creatine phosphate	50 ^j	Ca	71,000 ^k	1.0 ×10 ⁸ †	7.10×10 ⁶	— (<0.00001)
		Mg	25,000 ^k	1.0 ×10 ⁵ †	2,500	1.92 (0.039)
Inorganic phosphate	2 ^j	Ca	55,000 ⁿ	1.0 ×10 ⁸ †	5.50×10 ⁶	— (<0.00001)
		Mg	36,000 ⁿ	1.0 ×10 ⁵ †	3,600	0.054 (0.027)

The table lists typical values used for the model calculations. Temperature 20°C, pH 7.0 and [K⁺]_i=140 mM (ionic strength=0.17 M). Column 2 gives total concentration of the sites expressed as mmol/l cytoplasmic water (see Baylor *et al.* [45]). *Each molecule has two Ca²⁺-Mg²⁺ binding sites that are assumed to be independent. If the value of either k_{+1} or k_{-1} was not available, the value was calculated by using the relationship $K_D=k_{-1}/k_{+1}$ for columns 4–6. † k_{+1} was assumed as Robertson *et al.* [85]. Column 7 gives concentrations of the metal-site complexes ([metal-site]) for resting [Ca²⁺]_i = 0.1 μ M and resting [Mg²⁺]_i = 1 mM; fractional occupancies of the sites (= [metal-site]/[total site]) are also shown in parentheses. ^a Baylor *et al.* [45]. ^b Potter and Gergely [106]. ^c Johnson *et al.* [107]. ^d Calculated by assuming that the weight ratio of myosin (48 kD) and actin (4.2 kD) is 3 : 1 (60 and 20% of myofibrillar proteins), and the molar ratio of actin and troponin is 7 : 1 (see Ebashi *et al.* [108]). ^e Holroyde *et al.* [109]. ^f Bagshaw and Reed [110]. ^g Hou *et al.* [111]. ^h Ogawa and Tanokura [89]. ⁱ Ogawa and Tanokura [112]. ^j Godt and Maughan [9]. ^k Godt and Lindley [64]. ^l Fabiato and Fabiato [91]. ^m Assumed to be 100-fold lower than k_{+1} for Ca²⁺ [113]. ⁿ Martell and Smith [114].

bound to troponin and myosin is excluded as fixed (nondiffusible) Mg²⁺ because of high-affinity, the total diffusible [Mg²⁺] in the cytoplasm will be 8.9 mM. This value lies in the range of reported values in frog muscles (6.5 to 14.8 mM, see Godt and Maughan [9]). It can also be calculated that cytoplasmic Mg²⁺ buffering capacity, defined as the ratio of the change in total cytoplasmic [Mg²⁺] and the change in [Mg²⁺]_i, is 4.0. The difference compared with the lower value of about 2 calculated by Westerblad and Allen [74] is mostly due to the difference in the creatine phosphate concentration (50 mM assumed here versus 25 mM assumed for mouse muscle by Westerblad and Allen [74]) used for calculations.

Results of the simulation

Figure 2 shows the results of the model calculation with standard parameters for binding sites (Table 3). Traces without asterisks are for single stimulus, and those with asterisks are for repetitive stimuli. The Ca²⁺ transients derived from fura-dextran fluorescence signals with correction for the kinetic delay of the Ca²⁺-indicator reaction [32] were used as a driving function (Δ [Ca²⁺], top traces of the left column). Because fura-2 and other tetracarboxylate dyes gener-

ally have large selectivity for Ca²⁺ over Mg²⁺, the contribution of the effect of [Mg²⁺]_i changes on the fluorescence signals from fura dextran is probably negligible. The Ca²⁺ transient stimulated by an action potential has a similar time course and peak amplitude as those reported using low-affinity indicators; time to peak, 5.3 ms; half width, 9.7 ms; and peak amplitude, 10.3 μ M above the resting level. Following the Ca²⁺ transient, as clearly seen in Fig. 2, parvalbumin binds a large amount of Ca²⁺ (upward deflection of Δ [Ca-Parv]) and releases a significant amount of Mg²⁺ (downward deflection of Δ [Mg-Parv]). Released Mg²⁺ is buffered mainly by creatine phosphate (upward deflection of Δ [Mg-PCr]), as suggested by earlier studies [18, 81]. ATP initially releases Mg²⁺ on Ca²⁺ binding, but takes up Mg²⁺ at a later time (see reversal of (Δ [Mg-ATP])) because [Ca²⁺]_i decays to a low level and [Mg²⁺]_i slowly rises. Traces for inorganic phosphate (Δ [Ca-P_i] and Δ [Mg-P_i], not shown) look almost exactly like those for creatine phosphate with a greatly reduced amplitude; Δ [Ca-P_i] scaled by a factor of 19 and Δ [Mg-P_i] scaled by a factor of 35 match Δ [Ca-PCr] and Δ [Mg-PCr], respectively, in all cases tested with standard constants for creatine phosphate (Table 3). These scaling factors are expected

Fig. 2. Modeling of competition between Ca^{2+} (A) and Mg^{2+} (B) following a single-action potential or a train of action potentials spaced 20 ms apart (*). Cytoplasmic Ca^{2+} transients ($\Delta[\text{Ca}^{2+}]$) stimulated at time zero were derived from fluorescence signals of fura-dextran recorded in a frog fast muscle fiber (17°C, sarcomere length 3.8 μm , diameter 120 μm). kinetic delay of Ca^{2+} -indicator reaction was corrected, as previously described [61], by using the indicator's association rate constant of $3.2 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ and dissociation rate constant of 80 s^{-1} . From the Ca^{2+} transient, the Ca^{2+} and Mg^{2+} bindings to site X were calculated by numerically solving simultaneous differential equations of the form $d[\text{CaX}]/dt = k_{+1}(\text{Ca:X}) \cdot [\text{Ca}^{2+}] \cdot [\text{X}] - k_{-1}(\text{Ca:X}) \cdot [\text{CaX}]$,

$d[\text{MgX}]/dt = k_{+1}(\text{Mg:X}) \cdot [\text{Mg}^{2+}] \cdot [\text{X}] - k_{-1}(\text{Mg:X}) \cdot [\text{MgX}]$, using the rate constants listed in Table 3. The sum of changes in Mg^{2+} binding gives Mg^{2+} transient ($\Delta[\text{Mg}^{2+}]$): $d[\text{Mg}^{2+}]/dt = -\Sigma(d[\text{MgX}]/dt)$. Resting levels of $[\text{Ca}^{2+}]$ and $[\text{Mg}^{2+}]$ were set at 0.1 μM and 1.0 mM, respectively. $\Delta[\text{Ca-P}_i]$ and $\Delta[\text{Mg-P}_i]$ are not shown. Trop, high-affinity sites of troponin; Parv, parvalbumin sites; Pcr, creatine phosphate site. Note that troponin low-affinity (Ca^{2+} -specific) sites are not included in the calculation (see text). If they are included ($k_{+1} = 0.575 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$; $k_{-1} = 115 \text{ s}^{-1}$ [45]), the peak of $\Delta[\text{CaX}]$ for the troponin low-affinity sites would be 189 μM with a time to peak of 8 to 9 ms after a single stimulus.

from the 25-fold difference in the amount and from the small difference in affinities for both Ca^{2+} and Mg^{2+} between inorganic phosphate and creatine phosphate.

At 120 ms after a single stimulus (at the end of the traces in Fig. 2), $[\text{Mg}^{2+}]_i$ increases by 44 μM from the baseline ($\Delta[\text{Mg}^{2+}]$). This value (44 μM) is similar to values estimated in cut fibers containing antipyrylazo III, on average 47 μM [81]. This similarity may be somewhat fortuitous, since cytoplasmic concentrations of Ca^{2+} - Mg^{2+} buffers are likely to be different between cut and intact fibers; for example, the internal solution used by Irving *et al.* [81] contained only 20 mM of creatine phosphate.

With repetitive stimuli separated by 20 ms, the Ca^{2+} transient does not summate, but the changes in concentrations of Mg^{2+} -bound sites and consequently the

Mg^{2+} transient do summate (right top trace with an asterisk in Fig. 2). The results of the calculation with 50 stimuli are shown in an extended time base in Fig. 3 (traces without asterisks). At the end of the 1 s stimulation, $\Delta[\text{Mg}^{2+}]$ reaches 0.17 mM and decays very slowly, and $[\text{Ca}^{2+}]_i$ rapidly falls to a quasi-steady level (top traces in Fig. 3). A large amount of Mg^{2+} is dissociated from parvalbumin (peak $\Delta[\text{Mg-Parv}] = -0.47 \text{ mM}$) with some additional Mg^{2+} released from troponin high-affinity sites (peak $\Delta[\text{Mg-Trop}] = -0.10 \text{ mM}$) in exchange with Ca^{2+} . Most of the released Mg^{2+} is bound to creatine phosphate (peak $\Delta[\text{Mg-PCr}] = +0.31 \text{ mM}$) and ATP (peak $\Delta[\text{Mg-ATP}] = +0.10 \text{ mM}$). Myosin and inorganic phosphate (not shown) seem to contribute little as Ca^{2+} and Mg^{2+} buffers even during 1 s tetanus.

Because uncertainties still remain about the resting

Fig. 3. A longtime-base modeling of Ca²⁺ (A) and Mg²⁺ (B) competition during tetanus stimulated at 50 Hz for 1 s; a train of stimuli started at time 0. Traces were calculated, as described in the legend of Fig. 2, based on two different sets of resting [Ca²⁺] and Δ[Ca²⁺]. Model 1 used resting [Ca²⁺] of 0.1 μM and Δ[Ca²⁺] with the peak amplitude of ~10 μM, which had been calibrated as described in the legend of Fig. 2 from fluorescence signals of fura-dextran in a

frog fast-muscle fiber (17°C). Model 2 used resting [Ca²⁺] of 0.05 μM and Δ[Ca²⁺] scaled by a factor of two; the peak amplitude was ~20 μM. Traces calculated with model 2 are indicated by asterisks. Both models set resting [Mg²⁺] at 1.0 mM and used rate constants listed in Table 3. Δ[Ca-P_i] and Δ[Mg-P_i] are not shown. See legend of Fig. 2 for site abbreviations.

[Ca²⁺]_i level and amplitude of the Ca²⁺ transient (see above), the second set of model calculations (model 2) used a lower resting [Ca²⁺]_i (0.05 μM) and a larger peak amplitude of the Ca²⁺ transient (20.6 μM above the resting level) than those described in the previous sections (model 1). As shown in traces with asterisks in Fig. 3, the Mg²⁺ release from the sites on parvalbumin, troponin, and myosin is enhanced, as expected from the greater amount of Ca²⁺ binding to the sites. Mg²⁺ buffering of creatine phosphate, ATP, and inorganic phosphate (not shown) is also greater. The magnitude of Δ[Mg²⁺] (0.21 mM) is somewhat larger than the 0.17 mM obtained with model 1.

To further analyze the dependence of estimated magnitude of Δ[Mg²⁺] on the choice of parameters, additional runs of model 1 were carried out with one of the following three alterations of parameter values:

(1) k_{+1} values of parvalbumin for both Ca²⁺ and Mg²⁺ were increased by a factor of 10. Consequently, K_D values for Ca²⁺ and Mg²⁺ were respectively reduced to 0.013 and 115 μM. These values are similar

to those used by Gillis *et al.* [8]. Calculated Δ[Mg²⁺] had a peak of 0.24 mM.

(2) The apparent K_D value of ATP for Mg²⁺ was lowered to 75 μM, a value suggested by Godt and Lindley [64], by the reduction of k_{-1} to 75 s⁻¹. Calculated Δ[Mg²⁺] had a peak of 0.18 mM.

(3) The k_{-1} value of creatine phosphate for Mg²⁺ was increased twofold to set the K_D value at 50 mM, a value used by Fabiato and Fabiato [91]. The peak amplitude of calculated Δ[Mg²⁺] was 0.23 mM.

Thus either model 1 with any of these parameter selections or model 2 suggests that the peak change of [Mg²⁺]_i associated with 1 s tetanus is roughly 0.2 mM. However, if all the above changes were combined (i.e., the use of model 2 with simultaneous changes of (1), (2), and (3)), a much larger Mg²⁺ transient with 0.49 mM peak would be calculated.

The present simulation includes no metabolic changes that should occur during muscle contraction. However, the breakdown of creatine phosphate and the associated decrease in Mg²⁺ buffering capacity

appears too small to significantly enhance the Mg^{2+} transient. Tanokura and Yamada [92] reported a decrease in creatine phosphate concentration by 3.6 mM during 5 s tetanus. During 1 s tetanus (Fig. 3), the breakdown of creatine phosphate is expected to be smaller. Furthermore, the inorganic phosphate liberated in the process of creatine phosphate breakdown partly compensates for this small reduction in Mg^{2+} buffering capacity. Tanokura and Yamada [92] also reported cytoplasmic alkalization of 0.08 pH unit after 5 s tetanus; cytoplasmic pH changes in the present simulation (twitch and 1 s tetanus) are probably smaller and should make only a small contribution to the Mg^{2+} transients. It should be noted, however, that in fatigued muscles the breakdown of creatine phosphate (and ATP) and cytoplasmic acidification (because of lactate formation) may have a significant effect on $[Mg^{2+}]_i$ (e.g., Westerblad and Allen [74]).

There may be other sources of $[Mg^{2+}]_i$ changes that are not considered in the present simulation. For example, it has been suggested that during Ca^{2+} release from the SR, about 20% of the charge carried by Ca^{2+} is compensated by a counter-ion movement of Mg^{2+} [84]. With this movement from the cytoplasm to the SR lumen, $[Mg^{2+}]_i$ is expected to decrease transiently in the early phase after stimulation, though it will be partially compensated by the Mg^{2+} released to the free pool from ATP. The $[Mg^{2+}]_i$ change resulting from the counter-ion flux, however, has not been experimentally observed and is not included in the model calculations. It is also possible that intracellular Mg^{2+} binding sites that are still unidentified significantly contribute to the Mg^{2+} transient. Further experiments are required to resolve these uncertainties.

Conclusion

From the recent results accumulated in skeletal muscle fibers, it is likely that $[Ca^{2+}]_i$ very briefly changes from the resting level of ~ 0.05 – $0.1 \mu M$ to 10 – $20 \mu M$ following propagation of a single action potential. On the other hand, $[Mg^{2+}]_i$ seems to be well buffered around the resting level of about 1.0 mM. According to the model based on the likely estimates of resting $[Ca^{2+}]_i$, resting $[Mg^{2+}]_i$, and the Ca^{2+} transients, about 70% of Mg^{2+} released from parvalbumin and troponin in exchange with Ca^{2+} is buffered by creatine phosphate and ATP. The ~ 0.2 mM increase in $[Mg^{2+}]_i$ predicted for the large and sustained elevation of $[Ca^{2+}]_i$ should be confirmed experimentally to test the validity of the current model, and further studies are required to elucidate the physiological significance of the $[Mg^{2+}]_i$ change.

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REFERENCES

1. Endo M: Calcium release from the sarcoplasmic reticulum. *Physiol Rev* 57: 71–108, 1977
2. Ebashi S: Regulatory mechanism of muscle contraction with special reference to the Ca^{2+} -troponin-tropomyosin system. *In: Essays in Biochemistry*, ed. Campbell PN and Dickens F, Academic Press, London, Vol 10, pp 1–36, 1974
3. Endo M, Tanaka M, and Ogawa Y: Calcium induced release of calcium from the sarcoplasmic reticulum of skinned skeletal muscle fibres. *Nature (New Biol)* 228: 34–36, 1970
4. Ford LE and Podolsky RJ: Regenerative calcium release within muscle cells. *Science* 167: 58–59, 1970
5. Schneider MF and Simon BJ: Inactivation of calcium release from the sarcoplasmic reticulum in frog skeletal muscle. *J Physiol (Lond)* 405: 727–745, 1988
6. Donaldson SKB and Kerrick WGL: Characterization of the effects of Mg^{2+} on Ca^{2+} - and Sr^{2+} -activated tension generation of skinned skeletal muscle fibers. *J Gen Physiol* 66: 427–444, 1975
7. Inesi G: Mechanism of calcium transport. *Annu Rev Physiol* 47: 573–601, 1985
8. Gillis JM, Thomason D, Lefèvre J, and Kretsinger RH: Parvalbumins and muscle relaxation: a computer simulation study. *J Muscle Res Cell Motil* 3: 377–398, 1982
9. Godt RE and Maughan DW: On the composition of the cytosol of relaxed skeletal muscle of the frog. *Am J Physiol* 254: C591–C604, 1988
10. Cannell MB and Allen DG: Model of calcium movements during activation in the sarcomere of frog skeletal muscle. *Biophys J* 45: 913–925, 1984
11. Baylor SM and Hollingworth S: Model of sarcomeric Ca^{2+} movements, including ATP Ca^{2+} binding and diffusion, during activation of frog skeletal muscle. *J Gen Physiol* 112: 297–316, 1998
12. Simon W, Ammann D, Oehme M, and Morf WE: Calcium-selective electrodes. *Ann NY Acad Sci* 307: 52–69, 1978
13. Blinks JR, Wier WG, Hess P, and Prendergast FG: Measurement of Ca^{2+} concentrations in living cells. *Prog Biophys Molec Biol* 40: 1–114, 1982
14. Weingart R and Hess P: Free calcium in sheep cardiac tissue and frog skeletal muscle measured with Ca^{2+} -selective microelectrodes. *Pflügers Arch* 402: 1–9, 1984
15. Konishi M and Kurihara S: The use of Ca^{2+} indicators. *In: Current Methods in Muscle Physiology: Advantages, Problems and Limitations*, ed. Sugi H, Oxford University Press, Oxford and New York, pp 305–328, 1998
16. Dorogi PL and Newmann E: Spectrophotometric determination of reaction stoichiometry and equilibrium constants of metallochromic indicators. II. The Ca^{2+} -arsenazo complexes. *Biophys Chem* 13: 125–131, 1981
17. Rios E and Schneider MF: Stoichiometry of the reac-

- tions of calcium with the metallochromic indicator dyes antipyrilazo III and arsenazo III. *Biophys J* 36: 607–621, 1981
18. Baylor SM, Chandler WK, and Marshall MW: Optical measurements of intracellular pH and magnesium in frog skeletal muscle fibres. *J Physiol (Lond)* 331: 105–137, 1982
19. Palade P and Vergara J: Stoichiometries of arsenazo III–Ca complexes. *Biophys J* 43: 355–369, 1983
20. Allen DG, Blinks JR, and Prendergast FG: Aequorin luminescence: relation of light emission to calcium concentration—a calcium-independent component. *Science* 195: 996–998, 1977
21. Allen DG and Blinks JR: The interpretation of light signals from aequorin-injected skeletal and cardiac muscle cells: a new method of calibration. *In: Detection and Measurement of Free Ca²⁺ in Cells*, ed. Ashley CC and Campbell AK, Elsevier/North Holland, Amsterdam, pp 159–174, 1979
22. Moore EDW: Properties of aequorin relevant to its use in the measurement of intracellular [Ca²⁺] in skeletal muscle fibers. A thesis for PhD (Faculty of the Graduate School of the University of Minnesota), 1986
23. Konishi M and Kurihara S: Effects of caffeine on intracellular calcium concentration in frog skeletal muscle fibres. *J Physiol (Lond)* 383: 269–283, 1987
24. Blatter LA and Blinks JR: Simultaneous measurement of Ca²⁺ in muscle with Ca electrodes and aequorin. Diffusible cytoplasmic constituent reduces Ca²⁺-independent luminescence of aequorin. *J Gen Physiol* 98: 1141–1160, 1991
25. Grynkiewicz G, Poenie M, and Tsien R: A new generation of Ca²⁺ indicators with greatly improved fluorescence properties. *J Biol Chem* 260: 3440–3450, 1985
26. Zhao M, Hollingworth S, and Baylor SM: Properties of tri- and tetracarboxylate Ca²⁺ indicators in frog skeletal muscle fibers. *Biophys J* 70: 896–916, 1996
27. Konishi M, Olson A, Hollingworth S, and Baylor SM: Myoplasmic binding of fura-2 investigated by steady-state fluorescence and absorbance measurements. *Biophys J* 54: 1089–1104, 1988
28. Uto A, Arai H, and Ogawa Y: Reassessment of fura-2 and the ratio method for determination of intracellular Ca²⁺ concentrations. *Cell Calcium* 12: 29–37, 1991
29. Baker AJ, Brandes R, Schreur JHM, Camacho SA, and Wiener MW: Protein and acidosis alter calcium-binding and fluorescence spectra of the calcium indicator indo-1. *Biophys J* 67: 1646–1654, 1994
30. Harkins AB, Kurebayashi N, and Baylor SM: Resting myoplasmic free calcium in frog skeletal muscle fibers estimated with fluo-3. *Biophys J* 65: 865–881, 1993
31. Kurebayashi N, Harkins AB, and Baylor SM: Use of fura red as an intracellular calcium indicator in frog skeletal muscle fibers. *Biophys J* 64: 1934–1960, 1993
32. Konishi M and Watanabe M: Resting cytoplasmic free Ca²⁺ concentration in frog skeletal muscle measured with fura-2 conjugated to high molecular weight dextran. *J Gen Physiol* 106: 1123–1150, 1995
33. Klein MG, Simon BJ, Szucs G, and Schneider MF: Simultaneous recording of calcium transients in skeletal muscle using high- and low-affinity calcium indicators. *Biophys J* 53: 971–988, 1988
34. Suda N and Kurihara S: Intracellular calcium signals measured with fura-2 and aequorin in frog skeletal muscle fibers. *Jpn J Physiol* 41: 277–295, 1991
35. Li Q, Altschuld RA, and Stokes BT: Quantitation of intracellular free calcium in single adult cardiomyocytes by fura-2 fluorescence microscopy: calibration of fura-2 ratios. *Biochem Biophys Res Commun* 147: 120–126, 1987
36. Head SI: Membrane potential, resting calcium and calcium transients in isolated muscle fibres from normal and dystrophic mice. *J Physiol (Lond)* 469: 11–19, 1993
37. Carroll S, Klein MG, and Schneider MF: Calcium transients in intact rat skeletal muscle fibers in agarose gel. *Am J Physiol* 269: C28–C34, 1995
38. Konishi M and Watanabe M: Molecular size-dependent leakage of intracellular molecules from frog skeletal muscle fibers permeabilized with β -escin. *Pflügers Arch* 429: 598–600, 1995
39. Westerblad H and Allen DG: The influence of intracellular pH on contraction, relaxation and [Ca²⁺]_i in intact single fibres from mouse muscle. *J Physiol (Lond)* 466: 611–628, 1993
40. Westerblad H and Allen DG: Intracellular calibration of the calcium indicator indo-1 in isolated fibers of *Xenopus* muscle. *Biophys J* 71: 908–917, 1996
41. Westerblad H and Allen DG: Methods for calibration of fluorescent calcium indicators in skeletal muscle fibers. *Biophys J* 66: 926–927, 1994
42. Baylor SM, Harkins AB, and Kurebayashi N: Response to Westerblad and Allen. *Biophys J* 66: 927–928, 1994
43. Baylor SM, Hollingworth S, Hui CS, and Quinta-Ferreira ME: Calcium transients from intact frog skeletal muscle fibers simultaneously injected with antipyrilazo III and azo 1. *J Physiol (Lond)* 365: 70P, 1985
44. Blinks JR, Rüdel R, and Taylor SR: Calcium transients in isolated amphibian skeletal muscle fibres: detection with aequorin. *J Physiol (Lond)* 277: 291–323, 1978
45. Baylor SM, Chandler WK, and Marshall MW: Sarcoplasmic reticulum calcium release in frog skeletal muscle fibres estimated from arsenazo III calcium transients. *J Physiol (Lond)* 344: 625–666, 1983
46. Maylie J, Irving M, Sizto NL, and Chandler WK: Calcium signals recorded from cut frog twitch fibers containing antipyrilazo III. *J Gen Physiol* 89: 83–143, 1987
47. Baylor SM and Hollingworth S: Fura-2 calcium transients in frog skeletal muscle fibres. *J Physiol (Lond)* 403: 151–192, 1988
48. Maylie J, Irving M, Sizto NL, and Chandler WK: Calcium signals recorded from cut frog twitch fibers containing tetramethylmurexide. *J Gen Physiol* 89: 145–176, 1987
49. Konishi M and Baylor SM: Myoplasmic calcium transients monitored with purpurate indicator dyes injected into intact frog skeletal muscle fibers. *J Gen Physiol* 97: 245–270, 1991
50. Hirota A, Chandler WK, Southwick PL, and Waggoner

- AS: Calcium signals recorded from two new purpurate indicators inside frog cut twitch fibers. *J Gen Physiol* 94: 597–631, 1989
51. Konishi M, Hollingworth S, Harkins AB, and Baylor SM: Myoplasmic calcium transients in intact frog skeletal muscle fibers monitored with the fluorescent indicator fura-2. *J Gen Physiol* 97: 271–301, 1991
 52. Baylor SM, Chandler WK, and Marshall MW: Use of metallochromic dyes to measure changes in myoplasmic calcium during activity in frog skeletal muscle fibres. *J Physiol (Lond)* 331: 139–177, 1982
 53. Southwick PL and Waggoner AS: Synthesis of purpurate-1,1'-diacetic acid PDAA tripotassium salt: a new calcium indicator for biological applications. *Org Prep Proc Int* 21: 493–500, 1989
 54. Raju B, Murphy E, Levy LA, Hall RD, and London RE: A fluorescent indicator for measuring cytosolic free magnesium. *Am J Physiol* 256: C540–C548, 1990
 55. Baylor SM, Hollingworth S, Hui CS, and Quinta-Ferreira ME: Properties of the metallochromic dyes arsenazo III, antipyrilazo III and azo-1 in frog skeletal muscle fibres at rest. *J Physiol (Lond)* 377: 89–141, 1986
 56. Hollingworth S and Baylor SM: The amplitude and time course of the myoplasmic free $[Ca^{2+}]$ transient in fast-twitch fibers of mouse muscle. *J Gen Physiol* 108: 455–469, 1996
 57. Natori R: The role of myofibrils, sarcoplasm and sarcolemma in muscle-contraction. *Jikeikai Med J* 1: 18–28, 1954
 58. Endo M and Iino M: Specific perforation of muscle cell membranes with preserved SR functions by saponin treatment. *J Muscle Res Cell Motil* 1: 89–101, 1980
 59. Westerblad H and Allen DG: The role of sarcoplasmic reticulum in relaxation of mouse muscle; effects of 2,5-di(*tert*-butyl)-1,4-benzohydroquinone. *J Physiol (Lond)* 474: 291–301, 1994
 60. Westerblad H, Lännergren J, and Allen DG: Slowed relaxation in fatigued skeletal muscle fibers of *Xenopus* and mouse. Contribution of $[Ca^{2+}]_i$ and cross-bridge. *J Gen Physiol* 109: 385–399, 1997
 61. Konishi M and Watanabe M: Steady state relation between cytoplasmic free Ca^{2+} concentration and force in intact frog skeletal muscle fibers. *J Gen Physiol* 111: 505–519, 1998
 62. Morgan DL, Claflin DR, and Julian FJ: The relationship between tension and slowly varying intracellular calcium concentration in intact frog skeletal muscle. *J Physiol (Lond)* 500: 177–192, 1997
 63. Claflin DR, Morgan DL, and Julian FJ: The effect of length on the relationship between tension and intracellular $[Ca^{2+}]$ in intact frog skeletal muscle fibres. *J Physiol (Lond)* 508: 179–186, 1998
 64. Godt RE and Lindley BD: Influence of temperature upon contractile activation and isometric force production in mechanically skinned muscle fibers of the frog. *J Gen Physiol* 80: 279–297, 1982
 65. Gupta RK, Gupta P, Yushok WD, and Rose ZB: On the noninvasive measurement of intracellular free magnesium by ^{31}P NMR spectroscopy. *Physiol Chem Phys Med NMR* 15: 265–288, 1983
 66. London RE: Methods for measurement of intracellular magnesium: NMR and fluorescence. *Annu Rev Physiol* 53: 241–258, 1991
 67. Nakayama S, Nomura H, and Tomita T: Intracellular-free magnesium in the smooth muscle of guinea pig taenia caeci: a concomitant analysis for magnesium and pH upon sodium removal. *J Gen Physiol* 103: 833–851, 1994
 68. Gupta RK, Benovic JL, and Rose ZB: Magnetic resonance studies of the binding of ATP and cations to human hemoglobin. *J Biol Chem* 253: 6165–6171, 1978
 69. Fabiato A: Calcium-induced release of calcium from the cardiac sarcoplasmic reticulum. *Am J Physiol* 245: C1–C14, 1983
 70. Gupta RK and Moore RD: ^{31}P NMR studies of intracellular free Mg^{2+} in intact frog skeletal muscle. *J Biol Chem* 255: 3987–3993, 1980
 71. Kushmerick MJ, Dillon PF, Meyer RA, Brown TR, Krisanda JM, and Sweeney HL: ^{31}P NMR spectroscopy, chemical analysis, and free Mg^{2+} of rabbit bladder and uterine smooth muscle. *J Biol Chem* 261: 14420–14429, 1986
 72. Hess P, Metzger P, and Weingart R: Free magnesium in sheep, ferret and frog striated muscle at rest measured with ion-selective micro-electrodes. *J Physiol (Lond)* 333: 173–188, 1982
 73. López JR, Alamo L, Caputo C, Vergara J, and DiPolo R: Direct measurement of intracellular free magnesium in frog skeletal muscle using magnesium-selective microelectrodes. *Biochim Biophys Acta* 804: 1–7, 1984
 74. Westerblad H and Allen DG: Myoplasmic free Mg^{2+} concentration during repetitive stimulation of single fibres from mouse skeletal muscle. *J Physiol (Lond)* 453: 413–434, 1992
 75. Silverman HS, Lisa FD, Hui RC, Miyata H, Sollot SJ, Hansford RG, Lakatta EG, and Stern MD: Regulation of intracellular free Mg^{2+} and contraction in single adult mammalian cardiac myocytes. *Am J Physiol* 266: C222–C233, 1994
 76. Tashiro M and Konishi M: Basal intracellular free Mg^{2+} concentration in smooth muscle cells of guinea pig tenia cecum: intracellular calibration of the fluorescent indicator fura-2. *Biophys J* 73: 3358–3370, 1996
 77. Csernoch L, Bernengo JC, Szentesi P, and Jacquemond V: Measurements of intracellular Mg^{2+} concentration in mouse skeletal muscle fibers with the fluorescent indicator mag-indo-1. *Biophys J* 75: 957–967, 1998
 78. Westerblad H and Allen DG: Myoplasmic Mg^{2+} concentration in *Xenopus* muscle fibres at rest, during fatigue and during metabolic blockade. *Exp Physiol* 77: 733–740, 1992
 79. Konishi M, Suda N, and Kurihara S: Fluorescence signals from the Mg^{2+}/Ca^{2+} indicator fura-2 in frog skeletal muscle fibers. *Biophys J* 64: 223–239, 1993
 80. Baylor SM, Hui CS, and Quinta-Ferreira ME: Isotropic components of antipyrilazo III signals from frog skeletal muscle fibers. In: *Calcium in Biological Systems*, ed. Rubin RP, Weiss G, and Putney JW Jr, Plenum Publishing Corp, New York, pp 339–349, 1985

81. Irving M, Maylie J, Sizto NL, and Chandler WK: Simultaneous monitoring of changes in magnesium and calcium concentrations in frog cut twitch fibers containing antipyrilazo III. *J Gen Physiol* 93: 585–608, 1989
82. Jacquemond V and Schneider MF: Effects of myoplasmic Mg²⁺ on calcium binding by parvalbumin and calcium uptake by the sarcoplasmic reticulum in frog skeletal muscle. *J Gen Physiol* 100: 115–135, 1992
83. Mobley BA and Eisenberg BR: Sizes of components in frog skeletal muscle measured by methods of stereology. *J Gen Physiol* 66: 31–45, 1975
84. Somlyo AV, Gonzalez-Serratos H, Shuman H, McClellan G, and Somlyo AP: Calcium release and ionic changes in the sarcoplasmic reticulum of tetanized muscle: an electron probe study. *J Cell Biol* 90: 577–594, 1981
85. Robertson SP, Johnson JD, and Potter JD: The time-course of Ca²⁺ exchange with calmodulin, troponin, parvalbumin, and myosin in response to transient increases in Ca²⁺. *Biophys J* 34: 559–569, 1981
86. Gosselin-Rey C, Piront A, and Gerday C: Polymorphism of parvalbumins and tissue distribution: characteristics of component I, isolated from red muscles of *Cyprinus carpio* L. *Biochim Biophys Acta* 532: 294–304, 1978
87. Gosselin-Rey C and Gerday C: Parvalbumins from frog skeletal muscle (*Rana temporaria* L.): isolation and characterization, structural modification associated with calcium binding. *Biochim Biophys Acta* 492: 53–63, 1977
88. Gerday C and Gillis JM: The possible role of parvalbumins in the control of contraction. *J Physiol (Lond)* 258: 96P–97P, 1976
89. Ogawa Y and Tanokura M: Steady-state properties of calcium binding to parvalbumins from bullfrog skeletal muscle: effects of Mg²⁺, pH, ionic strength, and temperature. *J Biochem* 99: 73–80, 1986
90. Potter JD, Johnson JD, Dedman JR, Schreiber WE, Mandel F, Jackson RL, and Means AR: Calcium-binding proteins: relationship of binding, structure, conformation and biological function. In: *Calcium-Binding Proteins and Calcium Function*, ed. Wasserman RH, Corrandino RA, Carafoli E, Kretsinger RH, MacLennan DH, and Siegel FL, Elsevier, North-Holland Inc, New York and Amsterdam, pp 239–250, 1977
91. Fabiato A and Fabiato F: Calculator programs for computing the composition of the solutions containing multiple metals and ligands used for experiments in skinned muscle cells. *J Physiol (Paris)* 75: 463–505, 1979
92. Tanokura M and Yamada K: Changes in intracellular pH and inorganic phosphate concentration during and after muscle contraction as studied by time-resolved ³¹P-NMR. *FEBS Lett* 171: 165–168, 1984
93. Tsien RY and Rink TJ: Neutral ion-selective microelectrodes for measurement of intracellular free calcium. *Biochim Biophys Acta* 599: 623–638, 1980
94. López JR, Alamo L, Caputo C, and DiPolo R: Determination of ionic calcium in frog skeletal muscle fibers. *Biophys J* 43: 1–4, 1983
95. Ballanyi K and Grafe P: Changes in intracellular ion activities induced by adrenaline in human and rat skeletal muscle. *Pflügers Arch* 411: 283–288, 1988
96. Iuzzo PA, Klein W, and Lehmann-Horn F: Fura-2 detected myoplasmic calcium and its correlation with contracture force in skeletal muscle from normal and malignant hyperthermia susceptible pigs. *Pflügers Arch* 411: 648–653, 1988
97. Cohen SM and Burt CT: ³¹P-nuclear magnetic relaxation studies of phosphocreatine in intact muscle: determination of intracellular free magnesium. *Proc Natl Acad Sci USA* 74: 4271–4275, 1977
98. Maughan D: Diffusible magnesium in frog skeletal muscle cells. *Biophys J* 43: 75–80, 1983
99. Alvarez-Leefmans FJ, Gamiño SM, Giraldez F, and González-Serratos H: Intracellular free magnesium measured with ion-selective micro-electrodes. *J Physiol (Lond)* 378: 461–483, 1986
100. Blatter LA: Intracellular free magnesium in frog skeletal muscle studied with a new type of magnesium-selective microelectrode: interactions between magnesium and sodium in the regulation of [Mg²⁺]_i. *Pflügers Arch* 416: 238–246, 1990
101. MacDermott M: The intracellular concentration of free magnesium in extensor digitorum longus muscles of the rat. *Exp Physiol* 75: 763–769, 1990
102. Ng LL, Davies JE, and Ameen M: Intracellular free-magnesium levels in vascular smooth muscle and striated muscle cells of the spontaneous hypertensive rat. *Metabolism* 41: 772–777, 1992
103. Buchli R, Meier D, Martin E, and Boesiger P: Assessment of absolute metabolite concentrations in human tissue by ³¹P MRS *in vivo*. Part II: Muscle, liver, kidney. *Magn Reson Med* 32: 453–458, 1994
104. Ryschon TW, Rosenstein DL, Rubinow DR, Niemela JE, Elin RJ, and Balaban RS: Relationship between skeletal muscle intracellular ionized magnesium and measurements of blood magnesium. *J Lab Clin Med* 127: 207–213, 1996
105. Irish AB, Thompson CH, Kemp GJ, Taylor DJ, and Radda GK: Intracellular free magnesium concentrations in skeletal muscle in chronic uremia. *Nephron* 76: 20–25, 1997
106. Potter JD and Gergely JG: The calcium and magnesium binding sites on troponin and their role in the regulation of myofibrillar adenosine triphosphate. *J Biol Chem* 250: 4628–4633, 1975
107. Johnson JD, Robinson DE, Robertson SP, Schwarz A, and Potter JD: Ca⁺⁺ exchange with troponin and the regulation of muscle contraction. In: *Regulation of Muscle Contraction: Excitation-Contraction Coupling*, ed. Grinnell AD and Brazier MAB, Academic Press Inc, New York, pp 241–259, 1981
108. Ebashi S, Endo M, and Ohtsuki I: Control of muscle contraction. *Q Rev Biophys* 2: 351–384, 1969
109. Holroyde MJ, Potter JD, and Solaro RJ: The calcium binding properties of phosphorylated and unphosphorylated cardiac and skeletal myosins. *J Biol Chem* 254: 6478–6482, 1979
110. Bagshaw CR and Reed GH: The significance of the slow dissociation of divalent metal ions from myosin “regulatory” light chains. *FEBS Lett* 81: 386–390, 1978

- 1977
111. Hou T-T, Johnson JD, and Rall JA: Parvalbumin content and Ca^{2+} and Mg^{2+} dissociation rates correlated with changes in relaxation rate of frog muscle fibres. *J Physiol (Lond)* 441: 285–304, 1991
 112. Ogawa Y and Tanokura M: Kinetic studies of calcium binding to parvalbumins from bullfrog skeletal muscle. *J Biochem* 99: 81–89, 1986
 113. Eigen M and Wilkins RG: The kinetics and mechanism of formation of metal complexes. *In: Mechanisms of Inorganic Reactions*, Advanced Chemistry Series 49, pp 55–67, 1965
 114. Martell AE and Smith RM: Critical Stability Constants, Plenum Publishing Corp, New York, Vol. 2, pp 283–284, 1974