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CALCIUM INDICATORS AND CALCIUM SIGNALLING IN SKELETAL MUSCLE FIBRES DURING EXCITATION- CONTRACTION COUPLING

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Abstract

During excitation-contraction coupling in skeletal muscle, calcium ions are released into the myoplasm by the sarcoplasmic reticulum (SR) in response to depolarization of the fibre's exterior membranes. Ca^{2+} then diffuses to the thin filaments, where Ca^{2+} binds to the Ca^{2+} regulatory sites on troponin to activate muscle contraction. Quantitative studies of these events in intact muscle preparations have relied heavily on Ca^{2+} -indicator dyes to measure the change in the spatially-averaged myoplasmic free Ca^{2+} concentration ($\Delta[\text{Ca}^{2+}]$) that results from the release of SR Ca^{2+} . In normal fibres stimulated by an action potential, $\Delta[\text{Ca}^{2+}]$ is large and brief, requiring that an accurate measurement of $\Delta[\text{Ca}^{2+}]$ be made with a low-affinity rapidly-responding indicator. Some low-affinity Ca^{2+} indicators monitor $\Delta[\text{Ca}^{2+}]$ much more accurately than others, however, as reviewed here in measurements in frog twitch fibres with sixteen low-affinity indicators. This article also examines measurements and simulations of $\Delta[\text{Ca}^{2+}]$ in mouse fast-twitch fibres. The simulations use a multi-compartment model of the sarcomere that takes into account Ca^{2+} 's release from the SR, its diffusion and binding within the myoplasm, and its re-sequestration by the SR Ca^{2+} pump. The simulations are quantitatively consistent with the measurements and appear to provide a satisfactory picture of the underlying Ca^{2+} movements.

Keywords

excitation-contraction coupling; calcium signalling; calcium indicators; skeletal muscle

1. Introduction

A change in the cytosolic Ca^{2+} concentration ($\Delta[\text{Ca}^{2+}]$) controls important electrical and metabolic events in many types of cells. For example, in striated muscle, an increase in $[\text{Ca}^{2+}]$ activates the cells' contractile machinery. Due to its widespread importance, intracellular Ca^{2+} signalling has been intensively studied in many laboratories. An important methodology in these studies has been the intracellular use of fluorescent Ca^{2+} indicator dyes. This review focuses on issues that arise when the experimental goal is to make a quantitatively accurate measurement of $\Delta[\text{Ca}^{2+}]$ in vertebrate skeletal muscle fibres with a

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Ca^{2+} indicator dye. These issues likely apply to measurements of $\Delta[\text{Ca}^{2+}]$ in a number of other cell types.

2. Background on studies of $\Delta[\text{Ca}^{2+}]$ in skeletal muscle fibres

In skeletal muscle fibres stimulated electrically, $\Delta[\text{Ca}^{2+}]$ arises primarily from the release of Ca^{2+} by the sarcoplasmic reticulum (SR) in response to depolarization of the transverse tubular (T-tubular) membranes. The release takes place at the triadic junctions (Franzini-Armstrong, 1970), which are specialized locations within each sarcomere, where the T-tubular and SR membranes come into close apposition (Fig. 1). Once released, Ca^{2+} diffuses within the myoplasm while also complexing with available binding sites on the myoplasmic Ca^{2+} buffers. The major Ca^{2+} buffers include: (i) troponin, which is an immobile buffer on the thin filaments whose Ca^{2+} regulatory sites control contractile activation; (ii) ATP, which is a mobile, rapidly-reacting Ca^{2+} buffer; (iii) parvalbumin, which is a less mobile, more slowly-reacting Ca^{2+} buffer; and (iv) the SR Ca^{2+} pump, which is an immobile buffer that is present at high density in the non-junctional SR membrane and which uses ATP to re-sequester Ca^{2+} during muscle relaxation. For a quantitative understanding of the myoplasmic Ca^{2+} movements that occur under physiological conditions, it is essential to have an accurate experimental estimate of $\Delta[\text{Ca}^{2+}]$ in normally functioning muscle fibres.

Studies of $\Delta[\text{Ca}^{2+}]$ in skeletal muscle have relied on three main experimental preparations: (i) intact fibres, which are single fibres or small bundles of fibres that have been dissected manually but are otherwise thought to be in essentially normal condition (e.g., Baylor et al., 1982a,b; Baylor and Hollingworth, 1988, 2003; Cannell and Allen, 1984; Caputo et al., 2004; Claflin et al., 1994; Close and Lannergren, 1984; Hollingworth et al., 1996; Miledi et al., 1977; Rome et al., 1996; Taylor et al., 1975; Turner et al., 1988; Westerblad and Allen, 1991, 1992); (ii) cut fibres, which are transected fibre segments that are mounted in a vaseline-gap voltage clamp apparatus that permits control of the membrane potential of a portion of the fibre segment (e.g., Delbono and Stefani, 1993; DiFranco et al., 2002; Escobar et al., 1994; Garcia and Schneider, 1993; Klein et al., 1988, 1991; Kovacs et al., 1979, 1983; Maylie et al., 1987a,b,c; Melzer et al., 1986; Palade and Vergara, 1982; Rios and Brum, 1987); (iii) enzyme-dissociated fibres, which are tendonless fibres that have been isolated by exposure of a whole muscle (or a bundle of fibres) to collagenase and/or related enzymes (e.g., Calderon et al., 2009, 2010; Caputo et al., 2004; Carroll et al., 1995, 1997; Collet et al., 1999; Head, 1993; Tutdibi et al., 1999; Woods et al., 2004). Intact and cut fibres are typically obtained from either frogs or rodents; to date, enzyme-dissociated fibres have usually been obtained from rodents. In all cases, a Ca^{2+} reporter molecule – most frequently, a fluorescent indicator dye – is introduced into the myoplasm to measure $\Delta[\text{Ca}^{2+}]$ elicited by membrane depolarization.

In frog twitch fibres stimulated by an action potential, spatially-averaged $\Delta[\text{Ca}^{2+}]$ is normally large and brief, both in cut fibres (Hirota et al., 1989) and in intact fibres (Hollingworth et al., 1996; Konishi and Baylor, 1991), with the peak amplitude of $\Delta[\text{Ca}^{2+}]$ estimated to be 10–25 μM and the full-duration at half maximum (FDHM) to be 7–12 ms (16–18°C). An accurate measurement of this large and brief $\Delta[\text{Ca}^{2+}]$ with a Ca^{2+} indicator requires that it be made with a low-affinity, rapidly-responding indicator (Baylor and Hollingworth, 1998; Hirota et al., 1989; Konishi and Kurihara, 1998; sections 4, 5 and 7). In the sections below, we review two types of studies carried out on skeletal muscle fibres stimulated by action potentials. The first concerns studies of $\Delta[\text{Ca}^{2+}]$ in intact frog fibres that reveal the strengths and weaknesses of the various low-affinity indicators that have been used to measure $\Delta[\text{Ca}^{2+}]$. The second concerns the use of one such indicator, fura-2 (Raju et al., 1989; sold commercially under the name mag-fura-2) to measure $\Delta[\text{Ca}^{2+}]$ in mouse fast-twitch fibres. The mouse measurements are used to illustrate how quantitative

information about $\Delta[\text{Ca}^{2+}]$ can be interpreted with a multi-compartment model of the sarcomere to estimate SR Ca^{2+} release, the binding and diffusion of Ca^{2+} within the sarcomere, and the re-sequestration of Ca^{2+} by the SR Ca^{2+} pump.

3. The family of low-affinity Ca^{2+} indicators

All of the indicator dyes discussed in this article are thought to react with Ca^{2+} according to a 1:1 binding reaction:

D and CaD represent the Ca^{2+} -free and Ca^{2+} -bound forms of the indicator, respectively; $K_{\text{D,Ca}}$ denotes the dissociation constant of the indicator for Ca^{2+} , which is equal to the ratio of the reverse (k_{off}) and forward (k_{on}) rate constants of the reaction. With many of the indicators, the metal binding site can also react with Mg^{2+} with an appreciable affinity; in this case, $K_{\text{D,Mg}}$ denotes the dissociation constant of the indicator for Mg^{2+} .

In vitro values of $K_{\text{D,Ca}}$ are usually measured in the absence of Mg^{2+} in a 0.1–0.2 M salt solution at a temperature of 16–22°C and a pH of ~7. For convenience below, we define a high-affinity indicator as one with $K_{\text{D,Ca}} < 1 \mu\text{M}$ and a low-affinity indicator as one with $K_{\text{D,Ca}} > 25 \mu\text{M}$. We also define $[\text{D}_\text{T}]$ as the total concentration of the indicator, and f_{CaD} as the fraction of the indicator in the Ca^{2+} -bound form ($= [\text{CaD}]/[\text{D}_\text{T}]$). For situations in which Mg^{2+} can be ignored, $[\text{D}_\text{T}] = [\text{D}] + [\text{CaD}]$; further, if the Ca^{2+} -indicator reaction is at equilibrium:

$$f_{\text{CaD}} = [\text{Ca}^{2+}] / ([\text{Ca}^{2+}] + K_{\text{D,Ca}}). \quad (1)$$

Column 1 of Table 1 lists the 16 low-affinity Ca^{2+} indicators that are discussed in this article and column 2 lists their *in vitro* values of $K_{\text{D,Ca}}$. Column 3 lists $K_{\text{D,Mg}}$ where this has been measured. In each case, $K_{\text{D,Mg}}$ is many-fold larger than $K_{\text{D,Ca}}$; this presumably also applies in cases where $K_{\text{D,Mg}}$ has not been measured. To our knowledge, this is a complete list of low-affinity 1:1 Ca^{2+} indicators that have been used to measure $\Delta[\text{Ca}^{2+}]$ in skeletal muscle fibres. All but one of these (DMPDAA) have been used in intact fibres, the results of which are the principal subject of this article. The list is divided into three categories according to the optical and chemical properties of the indicators.

Category A lists indicators of the purpurate family. These indicators absorb radiation primarily at visible wavelengths and undergo a fractionally large change in absorbance (ΔA) upon binding Ca^{2+} . In contrast to the other indicators, purpurate indicators have an affinity for Ca^{2+} that is quite low ($K_{\text{D,Ca}} > 700 \mu\text{M}$) and have little or no fluorescence in either the Ca^{2+} -free or Ca^{2+} -bound form.

Category B lists indicators of the tri-carboxylate family. The Ca^{2+} affinity of these indicators is 10- to 100-fold higher than that of the purpurate indicators; their affinity for Mg^{2+} is about two-orders of magnitude lower than their affinity for Ca^{2+} . All members of this family have a substantial absorbance and a measurable fluorescence; they also undergo a relatively large change in fluorescence (ΔF) upon binding either Ca^{2+} or Mg^{2+} . Mag-fluo-4 and magnesium orange have the unusual property that, although their ΔA associated with metal binding is fractionally small (cf. Fig. 2B,D, described below), their fluorescence upon binding either Ca^{2+} or Mg^{2+} increases many-fold (remarkably, ~100-fold in the case of mag-fluo-4; Invitrogen on-line catalog). Mag-fura-red, mag-fluo-4, and magnesium orange

absorb primarily visible radiation, whereas fura-2, mag-fura-5, and mag-indo-1 absorb primarily in the ultraviolet. The latter indicators, however, have a tail of absorbance at longer wavelengths (cf. Fig. 2A,C, described below), which permits a useful excitation of their fluorescence with visible radiation.

Fura-2, the first reported tri-carboxylate indicator, was initially described as a fluorescent Mg^{2+} indicator (Raju et al., 1989), and, indeed, it has been used to measure resting cytosolic free $[Mg^{2+}]$ ($[Mg^{2+}]_R$) in liver cells (Raju et al., 1989), skeletal muscle fibres (Konishi et al., 1993; Westerblad and Allen, 1992), and cardiac myocytes (Watanabe and Konishi, 2001). The measurement of $[Mg^{2+}]_R$ relies on fura-2's affinity for Ca^{2+} being sufficiently low that the indicator's resting fluorescence in the cytoplasm (F_R) is not normally influenced by resting free $[Ca^{2+}]$ ($[Ca^{2+}]_R$), which is typically $\leq 0.1 \mu M$. Nevertheless, fura-2's Ca^{2+} affinity is sufficiently high that, in cells that have a substantial Ca^{2+} transient during activity, a Ca^{2+} -related ΔF from the indicator can be recorded with a reasonable signal-noise ratio. For example, in frog intact fibres stimulated by action potentials, fura-2 fluorescence signals excited with either UV or visible wavelengths have been used to monitor $\Delta[Ca^{2+}]$ (Clafin et al., 1994; Hollingworth et al., 2009; Konishi et al., 1991, 1993; Zhao et al., 1996; sections 9 and 10). Because fura-2 and the other tri-carboxylate indicators have a non-negligible affinity for Mg^{2+} , an issue that requires examination is the extent to which their Ca^{2+} -related signals may be influenced by Mg^{2+} (section 11).

Fig. 2 shows examples of *in vitro* absorbance spectra of fura-2 (panels A and C) and mag-fluo-4 (panels B and D) obtained in the absence and presence of Ca^{2+} . The upper panels show absolute spectra and the lower panels show Ca^{2+} -difference spectra. With both indicators, the difference spectrum has a single shape whose amplitude increases with increasing concentrations of Ca^{2+} in the manner expected for a 1:1 binding reaction (not shown). The values of $K_{D,Ca}$ estimated from measurements of this type are $44 \mu M$ for fura-2 (Konishi et al., 1991) and $70 \mu M$ for mag-fluo-4 (Hollingworth et al., 2009).

Category C lists indicators of the tetra-carboxylate family (Grynkiewicz et al., 1985; Tsien, 1980, 1992). A major advantage of these indicators is that their affinity for Ca^{2+} is about five orders of magnitude larger than that for Mg^{2+} rather than the two orders of magnitude characteristic of the tri-carboxylate indicators; thus, Mg^{2+} -related effects with tetra-carboxylate indicators can usually be ignored. As with mag-fluo-4 and magnesium orange, most of these indicators (the exceptions being fura-5N and BTC) have (i) their primary absorbance in the visible region, with spectra that do not differ greatly between the Ca^{2+} -free and Ca^{2+} -bound forms; and (ii) a fluorescence that is relatively weak in the Ca^{2+} -free form but that increases many-fold upon binding Ca^{2+} . BTC also has its primary absorbance in the visible region, but it undergoes both a large ΔA and a large ΔF upon binding Ca^{2+} . Fura-5N, like other indicators that utilize the fura chromophore, has its primary absorbance in the ultraviolet, with peak absorbance wavelengths in the Ca^{2+} -free and Ca^{2+} -bound forms (376 nm and 338 nm, respectively) that are similar to those of other fura-based indicators (e.g., 370 and 328 nm, respectively, with fura-2; Fig. 2A). Also, like other fura-based indicators, fura-5N undergoes a fractionally large ΔA upon binding Ca^{2+} . Fura-5N, however, has an unusually long tail of absorbance in the visible region of the spectrum, which extends beyond 500 nm, whereas the corresponding tail for the other fura-based indicators extends only slightly beyond 400 nm (e.g., Fig. 2A). Fura-5N is also unusual in that its fluorescence is relatively weak in both the Ca^{2+} -free and Ca^{2+} -bound forms and, because of this, is not commercially available.

4. Advantages of low-affinity Ca^{2+} indicators

To estimate $\Delta[\text{Ca}^{2+}]$ with an indicator dye, intracellular measurements are usually made either of the indicator's F_R and activity-related ΔF , or A_R (resting absorbance) and activity-related ΔA . Calibration of the optical measurements in units of $\Delta[\text{Ca}^{2+}]$ is, in principle, a two-step process: (i) conversion of the measurements to Δf_{CaD} (the change in the fraction of the indicator in the Ca^{2+} -bound form); (ii) conversion of Δf_{CaD} to $\Delta[\text{Ca}^{2+}]$. For these steps, it is often assumed that the indicator's optical properties and $K_{D,\text{Ca}}$ measured *in vitro* in a simple salt solution apply *in vivo*, and that the kinetics of reaction 1 are not rate-limiting. (Some errors that can arise from these assumptions are discussed in the next section.) Use of a low-affinity indicator permits a further simplifying assumption, namely, that f_R (the value of f_{CaD} in the cell at rest) is zero. Δf_{CaD} can then be converted to $\Delta[\text{Ca}^{2+}]$ with the equation (cf. equation 1):

$$\Delta[\text{Ca}^{2+}] = K_{D,\text{Ca}} \cdot \Delta f_{\text{CaD}} / (1 - \Delta f_{\text{CaD}}). \quad (2)$$

Application of steps (i) and (ii) can be illustrated with fura-2 fluorescence measurements in frog muscle fibres obtained with an excitation wavelength of 410 ± 20 nm (Hollingworth et al., 1996; sections 9 and 10; see also Konishi et al., 1991). With this excitation, A_R and F_R are largely due to metal-free fura-2, and absorbance and fluorescence decrease when the indicator binds Ca^{2+} (cf. Fig. 2A). Δf_{CaD} can be estimated from the fibre's F_R and ΔF measurements with the relation:

$$\Delta f_{\text{CaD}} = -1.07 (\Delta F / F_R). \quad (3)$$

This follows from *in vitro* calibrations carried out at $[\text{Mg}^{2+}] = 0.6$ mM, for which f_{MgD} , the fraction of fura-2 in the Mg^{2+} -bound form, is 0.1, which is close to the average value reported in frog skeletal muscle fibres at rest (0.08; Konishi et al., 1993). In these calibrations, ΔF_{max} , the change in fura-2's fluorescence upon addition of saturating Ca^{2+} , is related to F , fura-2's fluorescence in the absence of Ca^{2+} , by $\Delta F_{\text{max}} = -F/1.07$.

The average peak Δf_{CaD} calculated with equation 3 in frog fibres stimulated by an action potential is 0.15 (Table 2, 16°C; Hollingworth et al., 1996, 2009). From equation (2), the peak of $\Delta[\text{Ca}^{2+}]$ is estimated to be 8.6 μM if the effective value of $K_{D,\text{Ca}}$ in the myoplasm is 49 μM , the value expected if $K_{D,\text{Ca}} = 44$ μM (in the absence of Mg^{2+}) and $f_{\text{MgD}} = 0.1$. If, however, the effective value of $K_{D,\text{Ca}}$ is two-fold larger, as proposed by Konishi et al. (1991) (see also section 5), peak $\Delta[\text{Ca}^{2+}]$ would be corresponding larger, ~ 17 μM .

The relation between $\Delta[\text{Ca}^{2+}]$ and Δf_{CaD} (equation 2) is non-linear and applies accurately to spatially-averaged measurements of Δf_{CaD} only if there are no large gradients in $\Delta[\text{Ca}^{2+}]$ within the measurement region. In skeletal muscle fibres, however, $\Delta[\text{Ca}^{2+}]$ varies widely in different regions of the sarcomere (Baylor and Hollingworth, 1998, 2007; Cannell and Allen, 1984; DiFranco et al., 2002; Gomez et al., 2006; Hollingworth et al., 2000; section 12); consequently, equation 2 will underestimate the peak of (spatially-averaged) $\Delta[\text{Ca}^{2+}]$. This occurs because (i) the quantity $\Delta f_{\text{CaD}}/(1 - \Delta f_{\text{CaD}})$, if evaluated within different regions of the sarcomere, will be close to Δf_{CaD} in regions of the sarcomere where $\Delta[\text{Ca}^{2+}]$ is small but larger than Δf_{CaD} in regions where $\Delta[\text{Ca}^{2+}]$ is large, and (ii) the contribution of $\Delta[\text{Ca}^{2+}]$ in the latter regions is under-weighted when equation 2 is applied to spatially-averaged measurements of Δf_{CaD} . One advantage of low-affinity indicators is that the error due to this effect is smaller than that with commonly-employed high-affinity indicators, because, with high-affinity indicators, intra-sarcomeric variations in f_{CaD} ($= f_R + \Delta f_{\text{CaD}}$) are expected to be

very large. The non-linear relation between $\Delta[\text{Ca}^{2+}]$ and f_{CaD} can lead to a another complication with a high-affinity indicator: when f_{CaD} is close to 1, a small error in the estimation of f_{CaD} will produce a large error in the estimated amplitude of $\Delta[\text{Ca}^{2+}]$ (Hirota et al., 1989; see also section 5).

Low-affinity indicators also have advantages based on the kinetics of their reaction with Ca^{2+} . For a 1:1 reaction, tau, the time constant with which Δf_{CaD} tracks $\Delta[\text{Ca}^{2+}]$ at any instant is given by (e.g., Kao and Tsien, 1988):

$$\tau = 1 / (k_{\text{on}}([\text{Ca}^{2+}]_{\text{R}} + \Delta[\text{Ca}^{2+}]) + k_{\text{off}}). \quad (4)$$

If $[\text{Ca}^{2+}]_{\text{R}} + \Delta[\text{Ca}^{2+}] \ll K_{\text{D,Ca}}$, this simplifies to $\tau = 1/k_{\text{off}}$. Thus, k_{off} sets an important limitation on how rapidly Δf_{CaD} can track $\Delta[\text{Ca}^{2+}]$ in the cytosol. With the high-affinity indicator fura-2, $K_{\text{D,Ca}}$ is $\sim 0.2 \mu\text{M}$ in a salt solution and k_{off} is $\sim 100 \text{ s}^{-1}$ (20–22 °C; Kao and Tsien, 1988; Naraghi, 1997); thus, $\tau \approx 5 \text{ ms}$ if $[\text{Ca}^{2+}]_{\text{R}} + \Delta[\text{Ca}^{2+}] \approx K_{\text{D,Ca}}$ and $\tau \approx 10 \text{ ms}$ if $[\text{Ca}^{2+}]_{\text{R}} + \Delta[\text{Ca}^{2+}] \ll K_{\text{D,Ca}}$. A delay of this size will distort estimation of the time course of a $\Delta[\text{Ca}^{2+}]$ signal having a FDHM of ca. 3–8 ms, as occurs in frog and mouse skeletal muscle fibres at 20–22 °C. For example, during the decay phase of $\Delta[\text{Ca}^{2+}]$, the time course of Δf_{CaD} with fura-2 would be predicted to lag the late decline of $\Delta[\text{Ca}^{2+}]$ with a time constant that varies between 5 and 10 ms (since $[\text{Ca}^{2+}]_{\text{R}}$ is $< 0.2 \mu\text{M}$). In fact, an even larger distortion of $\Delta[\text{Ca}^{2+}]$'s time course is expected since, at the viscosity of myoplasm, which is estimated to be about twice that of a simple salt solution (Kushmerick and Podolsky, 1969), the values of k_{on} and k_{off} are expected to be about half those in a salt solution. Thus, as $\Delta[\text{Ca}^{2+}]$ declines toward baseline, a delay in the range 10–20 ms is expected between $[\text{Ca}^{2+}]$ and Δf_{CaD} measured with fura-2. In contrast, with a low-affinity indicator, the delay between $\Delta[\text{Ca}^{2+}]$ and Δf_{CaD} should be much smaller because the increased value of $K_{\text{D,Ca}}$ of a low-affinity indicator arises primarily from an increase in the value of k_{off} (Naraghi, 1997). For example, if $K_{\text{D,Ca}} > 25 \mu\text{M}$, the delay between $\Delta[\text{Ca}^{2+}]$ and Δf_{CaD} is expected to be ~ 100 -fold smaller than calculated above, i.e., 0.3 ms.

Although other phenomena may interfere with the ability of a low-affinity indicator to measure $\Delta[\text{Ca}^{2+}]$ (sections 5, 10 and 11), it may reasonably be asserted that an accurate estimate of a $\Delta[\text{Ca}^{2+}]$ that has a large amplitude, a brief time course, and large spatial inhomogeneities, as occurs in skeletal muscle fibres stimulated by action potentials, can only be obtained with a low-affinity indicator. The main difficulty expected *a priori* with a low-affinity indicator is that Δf_{CaD} will be small, which will result in an optical change with a correspondingly small signal-noise ratio. Thus, selection of a low-affinity indicator that has practical utility under a variety of measurement circumstances may still require some tradeoff between the linearity and kinetic fidelity of Δf_{CaD} on the one hand vs. the amplitude and signal-to-noise ratio of Δf_{CaD} on the other.

5. Complications due to the intracellular environment

Accurate estimation of Δf_{CaD} and $\Delta[\text{Ca}^{2+}]$ (and, in the case of high-affinity indicators, f_{R} and $[\text{Ca}^{2+}]_{\text{R}}$) may be difficult in muscle fibres because, with most indicators, a substantial fraction of the indicator binds to intracellular constituents, including soluble and structural proteins (Baker et al., 1994; Baylor et al., 1986; Beeler et al., 1980; Harkins et al., 1993; Hirota et al., 1989; Hollingworth et al., 2009; Hove-Madsen and Bers, 1992; Konishi et al., 1988, 1991; Kurebayashi et al., 1993; Maylie et al., 1987a,b,c; Zhao et al., 1996). One consequence of this binding is that the apparent diffusion coefficient of the indicator in myoplasm (D_{app}) will be smaller than expected based simply on the indicator's molecular weight and the viscosity of myoplasm (cf. Kushmerick and Podolsky, 1969). Columns 2 and

3 of Table 2 give, for most of the indicators in Table 1, the measured value of D_{app} and the estimated percentage of indicator molecules that appears to be bound to immobile myoplasmic constituents (assumed here to be myoplasmic proteins). In intact fibres, the bound percentage varies from 34% with PDAA to 95% with BTC. Among the fluorescent indicators, fura-2 and mag-fura-5 have the smallest bound percentages, 54 and 57%, respectively.

Binding of indicator to intracellular proteins is important because binding can alter the properties of the Ca^{2+} -indicator reaction, including alterations in (i) absorbance spectra, fluorescence spectra, and fluorescence quantum efficiency, and (ii) the effective value of $K_{D,Ca}$ (Baker et al., 1994; Beeler et al., 1980; Harkins et al., 1993; Hove-Madsen and Bers, 1992; Konishi et al., 1988; Uto et al., 1991; Zhao et al., 1996). Because of (i), an error in the estimated amplitude of Δf_{CaD} may occur; because of (ii), a further error may follow when Δf_{CaD} is converted to $\Delta[Ca^{2+}]$.

In addition to the equilibrium complications that can arise due to the binding of indicator to protein, complications due to changes in the kinetics of the Ca^{2+} -indicator reaction may occur. For example, in frog twitch fibres containing the high-affinity indicators fura-2 or fluo-3, whose bound percentages are estimated to be 70 and 85%, respectively, the indicator appears to react with Ca^{2+} with several-fold smaller values of k_{on} and k_{off} than expected in the absence of indicator binding, both in intact fibres (Baylor and Hollingworth, 1988, 1988; Harkins et al., 1993) and in cut fibres (Klein et al., 1988; Pape et al., 1993). The presence of two pools of indicator – protein-free and protein-bound – that react with Ca^{2+} with different rate constants further complicates estimation of the time course of $\Delta[Ca^{2+}]$ from the time course of Δf_{CaD} . With a high-affinity indicator, the Δf_{CaD} of indicator molecules that are not protein-bound is expected to track $\Delta[Ca^{2+}]$ during the falling phase of $\Delta[Ca^{2+}]$ with a kinetic delay of perhaps 10–20 ms (20–22 °C; section 4), and, if protein-bound indicator reacts with Ca^{2+} with reduced values of k_{on} and k_{off} , the overall delay will be even larger. With a low-affinity indicator, however, the kinetic delay in the absence of protein-binding is expected to be small (e.g., <0.3 ms), and, even if binding of indicator to protein increased the delay several-fold, the overall delay is still not expected to be large.

Based on these complexities, as well as those discussed in sections 4 and 10, reliable estimates of $\Delta[Ca^{2+}]$ in cells with a large, brief, and spatially inhomogeneous Ca^{2+} transient would appear to require not only the use of a low-affinity Ca^{2+} indicator but also one whose properties have not had major alterations caused by the binding of indicator to intracellular proteins. Such alterations are likely to be less marked with indicators that have a smaller bound percentage, such as the purpurate indicators, fura-2, and mag-fura-5.

6. Multi-compartment model of Ca^{2+} movements within the sarcomere

As mentioned in section 4, the myoplasmic Ca^{2+} transient elicited by an action potential in skeletal muscle fibres varies widely in amplitude and time course in different regions of the sarcomere (see also section 13). This occurs because (i) the sites of SR Ca^{2+} release within the myoplasm are restricted to the locations of the triadic junctions, and (ii) the ability of Ca^{2+} to spread within the sarcomere is limited by the binding of Ca^{2+} to its buffers and by the rates of diffusion of Ca^{2+} and the mobile Ca^{2+} buffers. As an aid to understanding the processes that shape the amplitude and time course of $\Delta[Ca^{2+}]$, it is useful to have a multi-compartment model of the myoplasm of the type first described by Cannell and Allen (1984). Such a model, in combination with measurements of $\Delta[Ca^{2+}]$, permits an estimation of the SR Ca^{2+} release flux, of Ca^{2+} 's diffusion within the myoplasm, of Ca^{2+} binding to the major myoplasmic Ca^{2+} buffers, and of the re-sequestration of Ca^{2+} by the SR Ca^{2+} pump in response to a variety of stimuli. A model of this type also aids in understanding how the

affinity and reaction kinetics of a Ca^{2+} indicator affect the accuracy with which $\Delta[\text{Ca}^{2+}]$ can be estimated with the indicator (Baylor and Hollingworth, 1998). We describe here an 18-compartment model of a half-sarcomere that we have used for these purposes (a half-sarcomere suffices because the full sarcomere is symmetric about the m-line).

Fig. 3 diagrams the geometry of the model that we have used for frog twitch fibres. The myoplasmic space within a half-sarcomere of one myofibril is divided into 18 equal-volume compartments – six longitudinal by three radial. The radius of the myofibril is assumed to be $0.5\ \mu\text{m}$ and the half-sarcomere length to be $2.0\ \mu\text{m}$ (the latter corresponds to a full-sarcomere length of $4.0\ \mu\text{m}$, which is similar to the long sarcomere lengths typically used for Ca^{2+} measurements in our laboratory; see section 8). Each compartment contains the Ca^{2+} indicator (usually fura-2) and the major myoplasmic Ca^{2+} buffers – ATP, troponin, parvalbumin, and SERCA (sarco/endoplasmic reticulum Ca^{2+} ATPase) Ca^{2+} pumps – at appropriate concentrations (Table 3 and legend of Fig. 3). The use of an 18-compartment model appears to be sufficient for the issues analyzed here, as calculations with a more detailed model (100 compartments; Hollingworth et al., 2000) gave results similar to those described below with the 18-compartment model.

Fig. 4 shows the reaction schemes used to describe the binding of Ca^{2+} (and, in some cases, Mg^{2+} and H^+) to the Ca^{2+} buffers. The rate constants for these reactions are listed in Table 4. The reaction of Ca^{2+} with ATP (Fig. 4A) is a reduced reaction whose on-rate for Ca^{2+} binding is an effective rate in the presence of $1\ \text{mM}$ free Mg^{2+} . The reaction with parvalbumin (Fig. 4B) explicitly includes the competition between Ca^{2+} and Mg for parvalbumin's metal binding sites. The reaction with the troponin Ca^{2+} -regulatory sites (Fig. 4C) is a two-step reaction; the first Ca^{2+} ion binds with low-affinity and the second with high affinity, thus imparting an overall positive cooperativity (Baylor et al., 2002; Hollingworth et al., 2006), as reported in some experimental measurements (e.g., Fuchs and Bayuk, 1976; Grabarek et al., 1983). The reaction with the Ca^{2+} pump (Fig. 4D) involves competition among Ca^{2+} , Mg^{2+} and H^+ for the transport sites (Peinelt and Apell, 2002). The reaction with fura-2 (Fig. 4E) assumes that some of the indicator is bound to protein and that both protein-free and protein-bound indicator can react with Ca^{2+} (section 5) and contribute comparably to ΔF . The value of $K_{D,\text{Ca}}$ chosen for the reaction of Ca^{2+} with protein-free fura-2 is the same as applies in a simple salt solution at $f_{\text{MgD}} = 0.1$; however, to account for the higher viscosity of myoplasm, the forward and reverse rate constants for this reaction have been reduced about two-fold from the values expected in a salt solution. The value of $K_{D,\text{Ca}}$ for protein-bound fura-2 is assumed to be larger than that for protein-free fura-2, and the values of the forward and reverse rate constants smaller (Baylor and Hollingworth, 1998; Harkins et al., 1993; Konishi et al., 1988).

For each compartment, a set of differential equations is specified that describes (i) the reactions of Ca^{2+} with its buffers, and (ii) the diffusion of free Ca^{2+} and the mobile Ca^{2+} buffers across compartment boundaries. Table 3 gives the relevant diffusion coefficients and the values of $[\text{Ca}^{2+}]_R$, $[\text{Mg}^{2+}]_R$, and resting pH, which determine the initial occupancies of the Ca^{2+} buffer sites (see legend of Fig. 4). $[\text{Mg}^{2+}]$ and pH are assumed to remain constant during fibre activity.

The location of the SR Ca^{2+} release flux is at the periphery of the myofibril in the compartment closest to the z-line (downward arrow in Fig. 3). This location is consistent with that expected for frog fibres based on the location of their triadic junctions. Ca^{2+} pumping takes place in all compartments at the periphery of the myofibril (upward arrows in Fig. 3). This corresponds to the location of the longitudinal SR, which contains SERCA Ca^{2+} pumps throughout at a high density (Ferguson and Franzini-Armstrong, 1988; Scales and Inesi, 1976).

Modifications of this model for analysis of Ca^{2+} movements in mouse fast-twitch fibres are described below (section 13).

7. Simulated responses of Ca^{2+} indicators having different Ca^{2+} affinities

As discussed in section 4, the larger the value of an indicator's k_{off} and hence $K_{\text{D,Ca}}$, the more reliable is the indicator's ability to track the time course of $\Delta[\text{Ca}^{2+}]$. This expectation is confirmed in the simulations in Fig. 5. For these simulations, the value of $[\text{D}_\text{T}]$ was reduced from 100 μM (the standard value; Table 3) to 1 μM so that the small buffering effect of the indicator on $\Delta[\text{Ca}^{2+}]$ would be negligible. The SR Ca^{2+} release waveform elicited by an action potential (see legend of Fig. 5) was chosen so that the simulated $\Delta[\text{Ca}^{2+}]$ waveform would approximately match that measured in frog intact fibres (section 9). Fig. 5A shows three simulated Δf_{CaD} waveforms based on three different values of $K_{\text{D,Ca}}$ for the reaction of Ca^{2+} with protein-free indicator: 1, 50, and 1,000 μM . Fig. 5B shows the Δf_{CaD} waveforms scaled to have the same peak amplitude. In all three simulations, the reaction between Ca^{2+} and indicator follows the scheme in Fig. 4E; the rate constants for the first and third steps of the reaction (cf. Table 4E), however, were adjusted to cover the desired 1,000-fold range of $K_{\text{D,Ca}}$. To do so, the listed values of the reverse-rate constants for these reaction steps (denoted k_{-1} and k_{-2} , respectively, in Fig. 4E) were multiplied by 0.0204, 1.02, and 20.4, thereby generating $K_{\text{D,Ca}}$ values of 1, 50 and 1,000 μM for the first reaction step and values of 2.14, 107 and 2,140 μM for the third reaction step.

In Fig. 5, the amplitude of Δf_{CaD} becomes progressively smaller (panel A) and the time course of Δf_{CaD} becomes progressively briefer (panel B) as the values of $K_{\text{D,Ca}}$ increase. At $K_{\text{D,Ca}} = 1 \mu\text{M}$, the time course of Δf_{CaD} is very much slower than that of $\Delta[\text{Ca}^{2+}]$ itself (shown as the dashed trace in Fig. 5B). At $K_{\text{D,Ca}} = 50 \mu\text{M}$, the time course of Δf_{CaD} is much closer to that of $\Delta[\text{Ca}^{2+}]$ but still lags it slightly. At $K_{\text{D,Ca}} = 1,000 \mu\text{M}$, Δf_{CaD} 's time course essentially matches that of $\Delta[\text{Ca}^{2+}]$. These simulations confirm the expectation that, at progressively larger values of $K_{\text{D,Ca}}$, the time course of Δf_{CaD} becomes progressively closer to that of $\Delta[\text{Ca}^{2+}]$.

In the simulation in Fig. 5 at $K_{\text{D,Ca}} = 50 \mu\text{M}$, the values of FDHM of (spatially-averaged) $\Delta[\text{Ca}^{2+}]$ and (spatially-averaged) Δf_{CaD} are 5.07 and 8.24 ms, respectively. This difference is not due to kinetic limitations in the reactions of Ca^{2+} with indicator (reactions 1 and 3 in Table 4E). Rather, it is due to a partial saturation of the indicator with Ca^{2+} in regions of the sarcomere near the SR Ca^{2+} release site where $\Delta[\text{Ca}^{2+}]$ is large; for example, in the Ca^{2+} release compartment, peak $\Delta[\text{Ca}^{2+}] \approx 100 \mu\text{M}$ and peak $\Delta f_{\text{CaD}} \approx 0.5$. This broadens the time course of (local) Δf_{CaD} relative to that of (local) $\Delta[\text{Ca}^{2+}]$; for example, in the release compartment, FDHM is 3.1 ms for $\Delta[\text{Ca}^{2+}]$ and 5.1 ms for Δf_{CaD} . This conclusion is confirmed by a fourth simulation (not shown) in which the forward and reverse rate constants for the first and third reactions in Table 4E were increased 20-fold. In this circumstance, the values of $K_{\text{D,Ca}}$ for the first and third reactions remain unchanged but the reverse rate constants for these reactions now match the very rapid values in the simulation in Fig. 5B at $K_{\text{D,Ca}} = 1,000 \mu\text{M}$. With these changes, the simulated values of the peak and FDHM of (spatially-averaged) Δf_{CaD} , 0.139 and 8.40 ms, respectively, are essentially unchanged from those in Fig. 5B at $K_{\text{D,Ca}} = 50 \mu\text{M}$, 0.140 and 8.24 ms. Thus, for the time course of Δf_{CaD} to be as brief as that of $\Delta[\text{Ca}^{2+}]$, it is important that the peak of Δf_{CaD} remain small, thereby avoiding substantial local saturation of the indicator with Ca^{2+} .

8. Fibre techniques and experimental procedures

The experimental examples discussed below are drawn from intact fibre experiments on frog and mouse muscle carried out in our laboratory. The general procedures have been described (Baylor and Hollingworth, 1988, 2003; Hollingworth et al., 1996; Konishi et al., 1991; Zhao et al., 1996) as well as the procedures for recording Ca^{2+} -related signals from two indicators in the same fibre in response to the same $\Delta[\text{Ca}^{2+}]$ (Hollingworth et al., 2009; Konishi et al., 1991; Zhao et al., 1996). All indicators were used in their (nominally) membrane-impermeant form (non-AM-form), most commonly the potassium-salt form. The use of the membrane-impermeant form minimizes the possibility that the indicator will enter intracellular compartments, such as the sarcoplasmic reticulum and mitochondria, which would further complicate interpretation of the measurements. For example, a previous study in intact frog fibres that compared results with AM-loaded vs micro-injected indicators found that the amplitude of $\Delta F/F_R$ evoked by an action potential was usually smaller by 20 to 80% with AM loading than with micro-injection (Zhao et al., 1997).

Frog experiments

Intact single twitch fibres were dissected from leg muscles (ileofibularis and semitendinosus) of *R. temporaria* or *R. pipiens*. An isolated fibre was mounted on an optical bench apparatus in a temperature-controlled chamber with quartz windows at 16°C. The Ringer's solution bathing the fibre contained (in mM): 120 NaCl, 2.5 KCl, 1.8 CaCl_2 , and 5 PIPES (piperazine-N,N'-bis[2-ethane-sulfonic acid]) (pH, 7.1). To minimize movement artifacts in the optical recordings, fibres were stretched to a long sarcomere length (3.3 – 4.1 μm) and lowered onto pedestal supports. To further reduce fibre movement, in some experiments the Ringer's solution contained 5 μM BTS (N-benzyl-p-toluene sulfonamide), an inhibitor of myosin II ATPase (Cheung et al., 2002). Isometric tension responses were monitored in many experiments with a tension transducer (Sensonor, Horten, Norway) attached to one tendon of the preparation.

Mouse experiments

Bundles of fibres were dissected from the fourth head of extensor digitorum longus (EDL) muscles of Balb-C mice (7–14 weeks of age). This muscle consists almost exclusively of fast-twitch fibres (e.g., Hughes et al., 1989; Marechal et al., 1995; see also section 13). Care was taken during dissection to avoid disturbing the fibres on one side (surface) of the bundle, which were the fibres used for experimentation. The bundle was mounted on the optical bench apparatus in a mammalian Ringer's solution at 16 °C that was bubbled with oxygen and contained (in mM): 150 NaCl, 2 KCl, 2 CaCl_2 , 1 MgCl_2 and 5 HEPES (4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid) (pH, 7.4).

Indicators, injections, and electrical stimulation

PDAA was a gift from Dr. W. K. Chandler (Yale University, New Haven, CT), TMX was from Sigma-Aldrich (St. Louis, MO), and the remaining indicators were from Invitrogen Corp. (Carlsbad, CA). Either a single fibre (frog) or one fibre within a bundle of fibres (mouse) was carefully pressure-injected with an indicator that had been loaded into a micro-pipette at a concentration of 10–40 mM in either distilled water or in 100 mM KCl. Injections were usually completed within 1–5 minutes after fibre impalement; the fibre was then allowed to rest for a few minutes prior to onset of optical recording. Fibres were studied only if they gave stable, all-or-nothing ΔF or ΔA responses to brief supra-threshold shocks from a pair of extracellular electrodes positioned locally near the injection site. Fibres were typically studied for many tens of minutes to several hours after injection. The stimulus cathode was usually located 1–2 mm from the site of optical recording; thus the propagation time for the action potential to reach the recording site was usually 0.5–1 ms. In a number of

frog experiments, fibres were injected with two Ca^{2+} indicators, with injections carried out either (i) at two locations separated by 0.5 mm (if the optical properties of the indicators were sufficiently different that their signals could be readily separated; Hollingworth et al., 2009; Konishi et al., 1991; Zhao et al., 1996), or (ii) at two sites separated by 1.5–2 mm (if the optical properties of the indicators were not sufficiently different that their signals could be readily separated; Hollingworth et al., 2009).

Fluorescence measurements

In most experiments, visible radiation from a tungsten-halogen bulb was used to excite fluorescence from a ~300 μm length of fibre near the site of indicator injection; in some experiments, UV radiation from an arc lamp was used. The fluorescence excitation wavelengths were selected by interference filters of either narrow-band (± 5 nm) or wideband (± 15 nm) positioned between the light source and the fibre; fluorescence emission wavelengths were selected with a broad-band filter of longer wavelength (typical bandwidth, 50 – 100 nm) positioned between the fibre and the photodetector. Both F_R and ΔF were recorded in all experiments, and signal properties were usually analyzed as $\Delta F/F_R$. In all cases, the value of F_R was corrected for any non-indicator-related contribution to the measured resting intensity; this contribution was estimated from a fibre region removed from the injection site, where the indicator concentration was negligible.

Absorbance measurements

In a number of frog experiments, A_R and ΔA were measured by trans-illumination of the fibre with a small spot of quasi-monochromatic light selected by a narrow-band (± 5 nm) interference filter positioned between the light source and the fibre. A second narrow-band filter of similar band-pass was positioned between the fibre and the photodetector to block fluorescent light from reaching the detector. Both A_R and ΔA were corrected for contributions from the fibre's intrinsic absorbance based on comparable measurements at a wavelength that was beyond the absorbance band of the indicator (see, e.g., Konishi and Baylor, 1991).

Estimation of myoplasmic indicator concentrations

Values of $[D_T]$, which are referred to the myoplasmic water volume (Baylor et al., 1983), were estimated with Beer's law from indicator absorbance measurements in the fibre (A_R and/or ΔA), the measured fibre diameter, and an appropriate extinction coefficient that was estimated from the *in vitro* absorbance spectra. In some experiments with fluorescent indicators, absorbance and resting fluorescence measurements were made in the same fibre at essentially identical times, from which a calibration factor was obtained to relate $[D_T]$ to resting fluorescence intensity normalized by fibre cross-sectional area. This factor permitted estimation of $[D_T]$ in cases where resting fluorescence and fibre diameter, but not fibre absorbance, were known. The estimated values of $[D_T]$ were usually 2 mM (for the purpurate indicators) or 0.2 mM (for the remaining indicators), which is sufficiently small that $\Delta[\text{Ca}^{2+}]$ was not significantly perturbed due to Ca^{2+} buffering by the indicator (e.g., Baylor and Hollingworth, 2007; Hollingworth et al., 2009; Konishi et al., 1991).

Indicator diffusion coefficients and indicator binding

With most indicators, estimates were obtained for the indicator's apparent diffusion coefficient in myoplasm (D_{app} ; column 2 of Table 2). To do so, values of F_R , ΔF , A_R or ΔA were measured at different distances from the site of indicator injection approximately one hour after injection, and the amplitude vs. distance profile of the measurements was fitted with the one-dimensional diffusion equation (e.g., Konishi et al., 1991). From D_{app} and the indicator's molecular weight, the empirical formula of Zhao et al. (1996) was used to

estimate the percentage of indicator molecules that are bound to immobile myoplasmic constituents (column 3 of Table 2).

9. Simultaneous comparison of myoplasmic Ca^{2+} signals from PDAA and fura-2

Fig. 6 compares Δf_{CaD} signals from PDAA and fura-2 in a frog twitch fibre that was injected with both indicators and stimulated to give an action potential. The measurements were made from the same fibre region at essentially identical times, so that the measurements reflect the indicators' responses to the same $\Delta[\text{Ca}^{2+}]$. The values of FDHM of Δf_{CaD} are 6.3 ms for PDAA and 9.3 ms for fura-2. These are similar to the simulated values of Δf_{CaD} in Fig. 5 at $K_{\text{D,Ca}} = 1,000$ and $50 \mu\text{M}$ (for protein-free indicator), namely, 5.3 and 8.2 ms, respectively. Since the $K_{\text{D,Ca}}$ values of 1,000 and $50 \mu\text{M}$ are similar to those expected for protein-free PDAA and fura-2 in myoplasm ($870\text{--}950 \mu\text{M}$ and $49 \mu\text{M}$, respectively), the measurements and simulations are in general agreement regarding effects of $K_{\text{D,Ca}}$ on the time course of Δf_{CaD} .

The relative amplitudes of Δf_{CaD} in Fig. 6 can also be compared with those in the simulations. The peak of Δf_{CaD} with fura-2 is 0.119 and that with PDAA is 0.0096; the ratio of these is 12.4. In the simulations, the peak value of Δf_{CaD} with a $K_{\text{D,Ca}}$ of $50 \mu\text{M}$ is 0.140 while that with a $K_{\text{D,Ca}}$ of $1,000 \mu\text{M}$ is 0.0117. Therefore, the peak Δf_{CaD} that is predicted for a $K_{\text{D,Ca}}$ of $49 \mu\text{M}$ (cf. Table 4E, reaction step 1) is $0.143 (= 0.140 \times 50/49)$, and that for a $K_{\text{D,Ca}}$ of $910 \mu\text{M}$ (the midpoint of the range in Table 1 for PDAA) is $0.0129 (= 0.0117 \times 1,000/910)$. The ratio of these predicted values is 11.1. Thus the relative amplitudes of the measured PDAA and fura-2 Δf_{CaD} signals are also in general agreement with the simulations.

Overall, these comparisons imply that the use of fura-2 and PDAA as myoplasmic Ca^{2+} indicators in skeletal muscle fibres will lead to similar conclusions about the amplitude and time course of $\Delta[\text{Ca}^{2+}]$ if the fura-2 measurement is interpreted with a multi-compartment model of the sarcomere. The advantage of PDAA is that, because its $K_{\text{D,Ca}}$ is very large, its peak Δf_{CaD} is small and the time course of its Δf_{CaD} almost exactly matches that of $\Delta[\text{Ca}^{2+}]$. Thus, with PDAA, a compartment model to estimate spatially-averaged $\Delta[\text{Ca}^{2+}]$ is superfluous, since $\Delta[\text{Ca}^{2+}]$ can be accurately estimated from Δf_{CaD} with equation 2. In contrast, although an approximate estimate of $\Delta[\text{Ca}^{2+}]$ from fura-2's Δf_{CaD} can be made with equation 2, a more accurate estimate can be obtained by simulation with a compartment model (see section 13). With both indicators, some uncertainty remains due to (i) possible alterations in the effective value of $K_{\text{D,Ca}}$ in myoplasm due to the binding of indicator to myoplasmic proteins (section 5) and (ii) possible interference from non- Ca^{2+} -related signals (sections 10 and 11).

The extra complexity of the compartment model is counterbalanced by several important advantages of fura-2 over PDAA. First, the amplitude of fura-2's Δf_{CaD} is about an order of magnitude larger than that of PDAA's; thus, fura-2's Δf_{CaD} is less sensitive to interference from components of $\Delta F/F_R$ that are not directly related to Δf_{CaD} , including movement artifacts and possible slow components of the type discussed in the next section. Second, because fura-2 is a fluorescent indicator and PDAA is an absorbant indicator, errors due to imperfect corrections for effects of intrinsic optical signals (which are larger for absorbance than fluorescence signals) are less important with fura-2 than PDAA. Third, again because fura-2 is fluorescent, measurements of its Δf_{CaD} are much more conveniently made; for example, fura-2 can be used in a small cell or in one fibre within a bundle of fibres without the need to trans-illuminate the cell or fibre with a small spot of light, as is required for absorbance measurements.

Finally, it should be noted that a compartment model is also expected to aid in the analysis of Δf_{CaD} signals from high-affinity indicators such as fura-2 and fluo-3. A general problem in this case, however, is that it is difficult to accurately characterize the substantial kinetic delay that is expected between Δf_{CaD} and $\Delta[\text{Ca}^{2+}]$ under intracellular conditions (sections 4 and 5). Thus, quantitative inferences about both the amplitude and time course of $\Delta[\text{Ca}^{2+}]$ from measurements with a high-affinity indicator will likely entail substantial uncertainties even if interpreted with a multi-compartment model.

10. Comparison of Ca^{2+} signals from fura-2 and other low-affinity fluorescent indicators

Comparisons of the time course of fura-2's ΔF with that of the ΔA or ΔF of other Ca^{2+} indicators give information about the ability of the other indicators to monitor the time course of $\Delta[\text{Ca}^{2+}]$. Comparative information of this type has been obtained in frog intact fibres for all of the indicators in Table 1 except for DMPDAA, which has only been used in cut fibres. However, the similarity of the FDHM of DMPDAA's ΔA signal elicited by an action potential (9.0 ms at 16 °C; Table 2) to that of PDAA's ΔA signal in cut and intact fibres (6.9 and 8.7 ms, respectively, at 16 °C) suggests that the ΔA of DMPDAA tracks the kinetics of $\Delta[\text{Ca}^{2+}]$ with reasonable fidelity. In contrast, use of TMX, the other purpurate indicator in Table 1, is problematic because TMX has a significant membrane permeability (Ogawa et al., 1980; Ohnishi, 1979). In both cut and intact fibres, the ΔA signal from TMX is biphasic, with an early component that monitors $\Delta[\text{Ca}^{2+}]$ and a later component (of opposite sign) that appears to reflect a decrease in $[\text{Ca}^{2+}]$ within the SR lumen (Maylie et al., 1987a; Pape et al., 2007). Because the time courses of the two TMX components overlap, estimation of the $\Delta[\text{Ca}^{2+}]$ waveform from the ΔA of TMX is problematic.

Fluorescent indicators whose $\Delta F/F_R$ time course is indistinguishable from fura-2's

With three of the tri-carboxylate indicators in Table 1 – mag-fura-5, mag-indo-1, and mag-fluo-4 – the time course of the $\Delta F/F_R$ signal elicited by an action potential is very similar to that of fura-2's (last two columns of Table 2); thus these indicators appear to track the time course of $\Delta[\text{Ca}^{2+}]$ as accurately as fura-2. The experiments in Fig. 7 were carried out to test this conclusion more rigorously in the case of mag-fluo-4. In these experiments, which are analogous to that in Fig. 6, fibres were micro-injected with both fura-2 and mag-fluo-4. The two superimposed traces in Fig. 7A show the action-potential evoked $\Delta F/F_R$ signals of the two indicators scaled to have the same peak amplitude. The time courses of these waveforms are essentially identical (see also legend of Fig. 7). Fig. 7B shows a similar experiment in which a fibre was stimulated by both a single action potential and a train of five action potentials at 100 Hz; again, the normalized $\Delta F/F_R$ time courses from the two indicators are in excellent agreement. These experiments confirm that fura-2 and mag-fluo-4 report similar information about the time course of $\Delta[\text{Ca}^{2+}]$. As discussed in section 11, calibration of the amplitude of $\Delta[\text{Ca}^{2+}]$ from the $\Delta F/F_R$ signal from mag-fluo-4 is subject to greater uncertainty than that from fura-2 due to a greater influence of $[\text{Mg}^{2+}]$ on F_R of mag-fluo-4.

Indicators that report misleading information about $\Delta[\text{Ca}^{2+}]$

With many of the other indicators in Table 2, the FDHM of the $\Delta F/F_R$ signal is noticeably larger than fura-2's – sometimes remarkably so. For example, with calcium-green-5N, the average value of FDHM is 36.7 ms whereas that with fura-2 is 10.6 ms. The conclusion that the time course of $\Delta F/F_R$ with calcium-green-5N is much slower than that with fura-2 was confirmed in double-injection experiments with these indicators. In the experiment of Fig. 8A, the FDHM of $\Delta F/F_R$ with calcium-green-5N is 36.8 ms while that with fura-2 is 10.2 ms. The large FDHM with calcium-green-5N might conceivably be explained, at least

in part, under the hypothesis that its Δf_{CaD} is much larger than furaptra's (cf. Fig. 5); however, this is unlikely since furaptra's $K_{\text{D,Ca}}$ in *in vitro* measurements is smaller than that of calcium-green-5N (44 vs. 63–85 μM ; Table 1). Another possible explanation is that, because of binding, indicator molecules may be preferentially located in regions of the sarcomere in which $\Delta[\text{Ca}^{2+}]$ changes more slowly than spatially-averaged $\Delta[\text{Ca}^{2+}]$ (cf. section 13). A more likely explanation, however, is that the $\Delta F/F_R$ of calcium-green-5N consists of at least two components: a fast component due to complexation of Ca^{2+} by the indicator and a slow component that is not directly related to Ca^{2+} complexation by the indicator. Slow components of this type have been observed previously with other Ca^{2+} indicators, including the dichroic component of the ΔA signal from the metallochromic indicators Arsenazo III and Dichlorophosphonazo III (Baylor et al., 1982a) and the second component of the ΔF signal from mag-fura-red (the polarity of which with 480 nm excitation is opposite to that of the Ca^{2+} component; Zhao et al., 1996). The presence of a major slow component in the ΔF signal of calcium-green-5N and mag-fura-red clearly makes these indicators unreliable monitors of $\Delta[\text{Ca}^{2+}]$.

Fig. 8B shows another double-injection experiment, in this case with furaptra and OGB-5N. The FDHM of $\Delta F/F_R$ with OGB-5N, 13.3 ms, is 40% larger than that with furaptra, 9.5 ms, which confirms the difference in time courses that is suggested in Table 2 by the average values of FDHM for these indicators (16.6 and 10.6 ms, respectively). Again, this difference likely arises from contamination of the ΔF signal of OGB-5N with a significant slow (non-calcium) component. An increase in the FDHM of $\Delta F/F_R$ relative to furaptra's FDHM was also observed in double-injection experiments with furaptra and the following indicators: magnesium orange, calcium-orange-5N, fluo-5N, fura-5N (Hollingworth et al., 2009; Zhao et al., 1996) and rhod-5N (unpublished results of Baylor and Hollingworth). The smallest such increase was observed with fura-5N, whose average value of FDHM was 36% larger than that with furaptra. Double-injection experiments with furaptra and BTC (the other tetra-carboxylate indicator in Table 1) were not carried out; however, BTC, in addition to its rather large value of FDHM (16.2 ms, Table 2), has a marked time-dependent change in its intracellular fluorescent properties that clearly makes BTC an unsuitable Ca^{2+} indicator for use in skeletal muscle (Zhao et al., 1996). Thus, we conclude that mag-fura-red, magnesium orange, and all of the tetra-carboxylate indicators in Table 1 are not reliable monitors of $\Delta[\text{Ca}^{2+}]$, due primarily to the presence of slow components of ΔF not directly related to Ca^{2+} complexation. Interestingly, with all nine of these indicators, a very large percentage of the indicator molecules appears to be bound to immobile intracellular constituents (75–95%; Table 2). This degree of binding undoubtedly increases the likelihood that unexpected indicator responses, including substantial non-calcium components, may arise in the intracellular environment.

In conclusion, of the 15 indicators in Table 1 that have been used in intact fibres, only five – PDAA, furaptra, mag-fura-5, mag-indo-1, and mag-fluo-4 – appear to monitor the kinetics of $\Delta[\text{Ca}^{2+}]$ in the expected way. Of these, PDAA gives the most reliable kinetic information about $\Delta[\text{Ca}^{2+}]$ when $\Delta[\text{Ca}^{2+}]$ is calculated from Δf_{CaD} with equation 2. With the other four indicators, use of equation 2 leads to a detectable kinetic error of the type described in connection with Figs. 5–6, which arises because $K_{\text{D,Ca}}$ is not sufficiently large to avoid substantial partial saturation of the indicator with Ca^{2+} in regions of the sarcomere where $\Delta[\text{Ca}^{2+}]$ is large. Thus, a more accurate quantitative interpretation of the Δf_{CaD} signals from these latter indicators is aided by a compartment model of the type described in section 6 (see also section 13).

11. Possible interference from $[Mg^{2+}]$ and $\Delta[Mg^{2+}]$

Of the low-affinity fluorescent indicators available for studies of $\Delta[Ca^{2+}]$ in skeletal muscle fibres, the previous section indicates that fura-2, mag-fura-2, mag-indo-1 and mag-fluo-4 are the indicators of choice. Because all of these are tri-carboxylate indicators whose values of $K_{D,Mg}$ are in the millimolar range (column 3 of Table 1), a drawback that applies to all is that they have a non-negligible sensitivity to Mg^{2+} . $[Mg^{2+}]_R$ is therefore expected to affect F_R and hence the conversion of $\Delta F/F_R$ to Δf_{CaD} . Also, because $\Delta[Mg^{2+}]$ is not entirely negligible in response to action potential stimulation (Baylor et al., 1982b, 1985; Irving et al., 1989), $\Delta[Mg^{2+}]$ is expected to make some contribution to ΔF .

With fura-2, the effect of $[Mg^{2+}]_R$ on F_R was previously estimated in intact frog fibres, in which f_{MgD} is ~ 0.1 (see equation 3 and related discussion in section 4). Similarly small values of f_{MgD} likely also apply to the other tri-carboxylate indicators. The effect of f_{MgD} on the estimation of Δf_{CaD} from $\Delta F/F_R$ will vary with the indicator, however. For example, with fura-2, F_R arises primarily from the metal-free form of the indicator, as the fluorescence of the Mg^{2+} -bound form of fura-2 is only about one-fifth that of the Mg^{2+} -free form (with 410–420 nm excitation; Konishi et al., 1991; Raju et al., 1989); in contrast, with mag-fluo-4, F_R arises primarily from the Mg^{2+} -bound form of the indicator, as the fluorescence of this form is ~ 100 -fold larger than that of the metal-free form (Invitrogen on-line catalog). (With both indicators, the effect of Ca^{2+} -bound indicator on F_R can be ignored because of the small value of $[Ca^{2+}]_R$ in skeletal muscle, $0.1 \mu M$.) Thus, with mag-fluo-4, F_R , and hence $\Delta F/F_R$, is likely to be substantially more sensitive to variations in $[Mg^{2+}]_R$ than with fura-2. Consequently, calibration of the amplitude of Δf_{CaD} , and hence of $\Delta[Ca^{2+}]$, from $\Delta F/F_R$ will likely prove to be more variable with mag-fluo-4 than with fura-2.

With the tri-carboxylate indicators, some interference in ΔF from $\Delta[Mg^{2+}]$ is expected at later times after stimulation, when a mass action exchange of Ca^{2+} for Mg^{2+} takes place on buffers such as parvalbumin and the SR Ca^{2+} pump (Fig. 4; see also next section). With fura-2, previous measurements in frog fibres indicate that ΔF elicited by an action potential returns to a small quasi-steady baseline offset by ~ 100 ms after stimulation. The average amplitude of ΔF during the time period 120–200 ms after stimulation, if expressed as a fraction of the peak ΔF , is $0.02 - 0.03$ (Hollingworth et al., 2009; Zhao et al., 1996). Calculations suggest that approximately half (or slightly less) of this quasi-steady ΔF appears to arise from a small maintained increase in $[Ca^{2+}]$, with the remainder due to a small maintained increase in $[Mg^{2+}]$ (Konishi et al., 1991). Similar maintained signals, with similar relative contributions from $\Delta[Ca^{2+}]$ and $\Delta[Mg^{2+}]$, likely also occur with the other tri-carboxylate indicators (Hollingworth et al., 2009; Zhao et al., 1996). Thus, studies of the late time course of $\Delta[Ca^{2+}]$ may be more accurately carried out with other indicators, for example, the high-affinity tetra-carboxylate indicators fura-2 and fluo-3 (e.g., Baylor and Hollingworth, 1988; Klein et al., 1991), which have a very high selectivity for Ca^{2+} over Mg^{2+} and for which uncertain kinetic delays between ΔF and $\Delta[Ca^{2+}]$ become unimportant on a slow time scale. Studies with these and other high-affinity indicators, however, are subject to the other caveats discussed in section 5.

12. Studies of $\Delta[Ca^{2+}]$ in mouse fast-twitch fibres

In recent years, Ca^{2+} indicator dyes have been widely used to study excitation-contraction coupling in mammalian skeletal muscle, particularly of rodents (e.g., Baylor and Hollingworth, 2003, 2007; Calderon et al., 2009, 2010; Capote et al., 2005; Caputo et al., 2004; Carroll et al., 1995, 1997; Chin and Allen, 1996; Collet et al., 1999; Delbono and Stefani, 1993; Garcia and Schneider, 1993; Gomez et al., 2006; Head, 1993; Hollingworth et

al., 1996, 2008; Turner et al., 1988, 1991; Ursu et al., 2005; Westerblad and Allen, 1991; Westerblad et al., 1993; Woods et al., 2004). Fig. 9 shows averaged results from four representative mouse experiments carried out in our laboratory, in which fura2/AM Ca^{2+} transients and isometric tension responses elicited by action potentials were measured in intact fast-twitch fibres from EDL muscles (16 °C). The records of Δf_{CaD} in the figure were obtained by scaling the $\Delta F/F_R$ signals (not shown) with equation 3. In response to a single action potential, the values of the peak and FDHM of Δf_{CaD} are 0.155 and 5.3 ms (Fig. 9A), which are similar to the average values observed in 13 experiments of this type, 0.160 ± 0.004 and 5.5 ± 0.3 ms (mean $\pm$ sem; Hollingworth et al., 1996,2008). Because our EDL fura2/AM transients reveal very little fibre-to-fibre variation, it is likely that they arise from a relatively homogeneous population of fibres (next section; see also Calderon et al., 2010, and Rome et al., 1996). Interestingly, the average amplitude of Δf_{CaD} in mouse fast-twitch fibres is similar to that in frog twitch fibres (0.15; column 4 of Table 2), but the FDHM of Δf_{CaD} is smaller than that in frog fibres (10.6 ms; column 6 of Table 2). We conclude that the amplitude of $\Delta[\text{Ca}^{2+}]$ is similar in mouse fast-twitch and frog twitch fibres but $\Delta[\text{Ca}^{2+}]$ is briefer in mouse fibres.

Fig. 9B shows Δf_{CaD} elicited by a 5-shock train of action potentials at 67 Hz. In response to the later action potentials in the train, Δf_{CaD} rises to about the same peak as that elicited by the first action potential; the decay of Δf_{CaD} from peak, however, becomes progressively slower with each successive action potential. The explanation of the progressively slower decay is that myoplasmic Ca^{2+} buffers, primarily troponin and parvalbumin, become increasingly saturated with Ca^{2+} with each successive stimulus; thus, the ability of these buffers to bind Ca^{2+} and lower free $[\text{Ca}^{2+}]$ becomes progressively reduced (Baylor and Hollingworth, 2003,2007; see also Melzer et al., 1986). Following termination of the stimulus train, Δf_{CaD} returns to a quasi-steady baseline offset whose amplitude during the period 150–250 ms is about 0.131 of the peak Δf_{CaD} . This offset is larger than that in Fig. 9A, where the quasi-steady amplitude of Δf_{CaD} during the period 120 to 200 ms is 0.046 of the peak Δf_{CaD} . [Note: these offset values are slightly uncertain due to possible contamination of ΔF with small movement artifacts.] As mentioned in the previous section, quasi-steady signals from fura2/AM are expected due to maintained increases in both $[\text{Ca}^{2+}]$ and $[\text{Mg}^{2+}]$.

13. Simulations of $\Delta[\text{Ca}^{2+}]$ in mouse fast-twitch fibres

To estimate $\Delta[\text{Ca}^{2+}]$ and the associated intra-sarcomeric Ca^{2+} movements from measurements like those in Fig. 9, the compartment model shown in Fig. 3 was adapted to mouse fast-twitch fibres. To do so, two changes were made to the geometry of the model. First, the radius of the myofibril was reduced from 0.5 to 0.375 μm , to reflect the somewhat smaller average size of the myofibrils in mouse fibres vs. frog fibres (Goldspink, 1970; Peachey and Eisenberg, 1978). Second, the location of Ca^{2+} release was moved from the first compartment at the periphery of the myofibril (Fig. 3) to the second compartment at the periphery. The latter compartment is offset $\sim 0.5 \mu\text{m}$ from z-line, which corresponds to the location of the triadic junctions in mammalian muscle (Brown et al., 1998; Eisenberg, 1983; Smith, 1966). This offset also agrees with the Ca^{2+} release location determined in enzyme-dissociated mouse fibres with confocal microscopy and the Ca^{2+} indicator OGB-5N (Gomez et al., 2006). The other parameters of the model remained the same as for frog twitch fibres (Tables 3–4).

In simulations of Ca^{2+} movements in fast-twitch EDL fibres, a question that arises concerns the concentration of the parvalbumin $\text{Ca}^{2+}/\text{Mg}^{2+}$ sites that should be included in the model. For the following reasons, our model includes a large concentration of parvalbumin sites, 1.5 mM. First, although some mouse EDL fibres contain small to negligible concentrations

of parvalbumin, the majority of fibres contain large parvalbumin concentrations (Ecob-Prince and Leberer, 1989; see also Fuchtbauer et al., 1991). The average parvalbumin concentration in mouse EDL muscle is reported to be 4.4 – 4.9 g/kg wet weight (Berquin and Labacq, 1992; Heizmann et al., 1982), which, if referred to the myoplasmic water volume, corresponds to an average $\text{Ca}^{2+}/\text{Mg}^{2+}$ site concentration of 1.26 – 1.40 mM (Baylor and Hollingworth, 2007). Second, as noted in the preceding section, our EDL Ca^{2+} transients reveal very little fibre-to-fibre variation, which is consistent with the idea that they arise from a homogeneous population of fibres; on a statistical basis, it is likely that these are parvalbumin-rich fibres. Third, our fibres are always obtained from the surface (superficial) region of the muscle (section 8), where fibres tend to have larger parvalbumin concentrations than average (Ecob-Prince and Leberer, 1989; see also Leberer and Pette, 1986). Fourth, in a preliminary electron microscopy study, fibre bundles from the region of the EDL muscle that we use for our experiments have been tentatively identified as type IIX/IIB based on the characteristic distribution and frequency of mitochondria (unpublished observations of Simona Boncompagni). The average mitochondrial content of these fibres is smaller than that of type IIA fibres in EDL muscle, which have also been tentatively identified. Because the EDL fibres that we have studied are not highly oxidative, they are likely to be enriched in parvalbumin (Fuchtbauer et al., 1991).

To simulate the SR Ca^{2+} release flux elicited by an action potential, the following empirical function was used (Baylor and Hollingworth, 1998):

$$\text{Ca}^{2+} \text{ flux}(t) = 0 \text{ if } t < T, = R \cdot [1 - \exp(-(t - T)/\tau_1)]^L \cdot \exp(-(t - T)/\tau_2) \text{ if } t \geq T. \quad (5)$$

The time-shift parameter T was set to 1.4 ms, which simulates the delay between the external shock that generates the action potential and the onset of Ca^{2+} release. L , τ_1 , and τ_2 were set to 5, 1.3 ms, and 0.5 ms, respectively, so that the FDHM of the release flux would be 1.6 ms, the value estimated in a single-compartment analysis of the fura-2 Δf_{CaD} signal in mouse fibres (Baylor and Hollingworth, 2003). The value of R was adjusted to give good agreement between the amplitude of the simulated and measured Δf_{CaD} waveform. For the 5-shock 67-Hz train, the release function used to simulate the later responses in the train had the same functional form as that in equation 5 except that the parameter T was increased successively by 15 ms for each subsequent action potential; for each successive release event, the value of R was again adjusted to give good agreement between the corresponding peak of Δf_{CaD} in the simulations and the measurements.

The upper traces in Fig. 10 show a superposition of the Δf_{CaD} measurements in Fig. 9 (noisy traces) and the simulated versions of these measurements (noise-free traces); the lower traces show the SR Ca^{2+} release waveforms used to drive the simulations. The good agreement between the simulated and measured Δf_{CaD} waveforms indicates that the multi-compartment model provides a reasonable overall description of the Ca^{2+} movements that underlie the Δf_{CaD} measurements. In response to the single action potential (panel A), the peak amplitude of the SR Ca^{2+} flux is 205 $\mu\text{M}/\text{ms}$ and the corresponding total concentration of released Ca^{2+} ($\Delta[\text{Ca}_T]$; referred to the myoplasmic water volume) is 349 μM . Most of this released Ca^{2+} is quickly bound by the myoplasmic Ca^{2+} buffers and thus does not appear as free Ca^{2+} ; according to the simulation, the peak of $\Delta[\text{Ca}^{2+}]$ is 16 μM (Fig. 11A, described below), which is only ~5% of $\Delta[\text{Ca}_T]$.

In Fig. 10B, the amplitude of the release fluxes in response to the second and subsequent action potentials in the train varies between 25 and 15% of the amplitude of the first release flux. These large reductions in flux are thought to be due to the process of “ Ca^{2+} inactivation of Ca^{2+} release”; specifically, the rise in myoplasmic free $[\text{Ca}^{2+}]$ caused by the

prior release(s) feeds back on the SR release system to inhibit the subsequent release(s). The features of this process in mouse fast-twitch fibres (Baylor and Hollingworth, 2007; Capote et al., 2005; Hollingworth et al., 1996) appear to be generally similar to those described in frog twitch fibres (Baylor and Hollingworth, 1988; Baylor et al., 1983; Jong et al., 1993, 1995; Schneider and Simon, 1988; Simon et al., 1991). A possible difference between mouse and frog concerns the time course with which the peak release flux elicited by one action potential recovers from Ca^{2+} inactivation of Ca^{2+} release. In both fibre types, the recovery appears to follow a single-exponential time course; however, the recovery time constant is estimated to be ~140 ms in mouse fibres (16 °C; Baylor and Hollingworth, 2007) vs. ~25 ms in frog fibres (14–15 °C; Jong et al., 1995).

In Fig. 11, each panel shows two versions of (spatially-averaged) $\Delta[\text{Ca}^{2+}]$. The noisy waveform was calculated with equation 2 from the corresponding measurement of (spatially-averaged) Δf_{CaD} (Figs. 9A,B) under the assumption that furaptra's effective value of $K_{\text{D,Ca}}$ in myoplasm is 96 μM , the value used previously to analyze furaptra Δf_{CaD} signals in frog fibres (Baylor and Hollingworth, 1998; Konishi et al., 1991). The noise-free waveform is $\Delta[\text{Ca}^{2+}]$ from the simulations of Fig. 10, obtained by taking the average of the individual $\Delta[\text{Ca}^{2+}]$ waveforms in the eighteen compartments. Overall, the simulated waveform is thought to be more accurate than the waveform calculated with equation 2. As is apparent in Fig. 11A, approximate agreement is found between the simulated amplitude of $\Delta[\text{Ca}^{2+}]$ elicited by one action potential and that calculated with equation 2 from the measurement of Δf_{CaD} ; as expected, the FDHM of $\Delta[\text{Ca}^{2+}]$ calculated with equation 2 is larger than that of the simulated $\Delta[\text{Ca}^{2+}]$ (see legend and section 7). In Fig. 11B, more substantial discrepancies appear at later times between the simulated $\Delta[\text{Ca}^{2+}]$ and that calculated from Δf_{CaD} with equation 2. As discussed in sections 4 and 7, it cannot be expected that (spatially-averaged) $\Delta[\text{Ca}^{2+}]$ can be accurately estimated with equation 2 from furaptra's Δf_{CaD} under experimental circumstances in which there are large and variable gradients in (local) $\Delta[\text{Ca}^{2+}]$. The discrepancies between the $\Delta[\text{Ca}^{2+}]$ waveforms in Fig. 11 emphasize the limitations of equation 2 – and hence the value of a compartment model – in the estimation of $\Delta[\text{Ca}^{2+}]$ with furaptra and other indicators with similar values of $K_{\text{D,Ca}}$.

Fig. 12 shows, for the simulation of Fig. 10A, spatially-resolved information (i.e., compartment information) for some of the constituents in the model. In each panel, the continuous traces show the maximum and minimum concentration changes found among all compartments for the following variables: $\Delta[\text{Ca}^{2+}]$; $\Delta[\text{CaParv}]$ (the change in the concentration of Ca^{2+} bound to parvalbumin); $\Delta[\text{CaPump}]$ (the change in the concentration of Ca^{2+} bound to the Ca^{2+} pump); and $\Delta[\text{CaTrop}]$ (the change in the concentration of Ca^{2+} bound to troponin); the last two changes include the sum of both the singly- and doubly-bound Ca^{2+} states of these buffers (Figs. 4C,D). The dashed trace in each panel shows the simulated spatially-averaged change for each of the variables. Fig. 12 reveals that $\Delta[\text{Ca}^{2+}]$, $\Delta[\text{CaParv}]$ and $\Delta[\text{CaPump}]$ have large differences in amplitude and/or time course in different regions of the sarcomere. In contrast, the differences in $\Delta[\text{CaTrop}]$ are much smaller. Similarly small differences in $\Delta[\text{CaTrop}]$ are also predicted to occur at physiological sarcomere lengths, e.g., 2.4 μm (Baylor and Hollingworth, 2007). Thus, the geometry of the sarcomere and the properties of the SR Ca^{2+} release flux appear to be well designed to achieve a rapid and nearly uniform binding of Ca^{2+} to the Ca^{2+} -regulatory sites on the thin filament. This, in turn, is expected to elicit a well-synchronized activation of the contractile response of the myofilaments.

14. Conclusions

This article has examined a number of issues that affect the accuracy of interpretations of optical signals from Ca^{2+} indicator dyes introduced into the cytosol. Many of these issues

have not reached the level of major concern in most previous studies of intracellular Ca^{2+} signalling with fluorescent Ca^{2+} indicators. The reasons for this are several-fold. First, many studies seek a qualitative answer to the biological question of interest – for example, is $\Delta[\text{Ca}^{2+}]$ important in the process under study or does $\Delta[\text{Ca}^{2+}]$ change as a result of some experimental maneuver. Second, the simplest way to monitor $\Delta[\text{Ca}^{2+}]$ qualitatively is usually with a high-affinity indicator that has been AM-loaded into a preparation, even if the information obtained is subject to interpretation difficulties. Third, the use of more than one Ca^{2+} indicator to confirm the accuracy of any particular measurement is a potentially open-ended activity. Fourth, analysis of spatially-averaged Ca^{2+} signals that arise from processes that involve large gradients in $\Delta[\text{Ca}^{2+}]$ – a situation that applies to most Ca^{2+} signalling events – entails considerable complexity. Hopefully, future studies in which quantitative interpretations of $\Delta[\text{Ca}^{2+}]$ and the underlying Ca^{2+} movements are important will further elucidate the issues explored here.

Finally, it should be acknowledged that obtaining quantitatively accurate information about Ca^{2+} signals under normal intracellular conditions is a challenging endeavor. In the case of skeletal muscle fibres, which are some of the largest and most experimentally accessible cells, several dozen Ca^{2+} -sensitive molecules have been used to date to study $\Delta[\text{Ca}^{2+}]$ during excitation-contraction coupling, including photoproteins, metallochromic indicators, purpurate indicators, and high- and low-affinity fluorescent indicators. While a great deal of valuable information has been obtained in these studies, it is remarkable that no Ca^{2+} indicator has yet been identified that reports an optical change elicited by an action potential that: (a) has an amplitude that represents at least a 5% change with respect to the resting level (e.g., $\Delta F/F_R \geq 0.05$ or $\Delta F/F_R \leq -0.05$), (b) tracks $\Delta[\text{Ca}^{2+}]$ with good kinetic fidelity, and (c) is free of interference from non- Ca^{2+} components, including interference from Mg^{2+} . It is hoped that future technological developments will rectify this situation.

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Non-standard abbreviations

A_R	indicator absorbance in the cytosol at rest
$[\text{Ca}^{2+}]$	the concentration of free Ca^{2+}
$[\text{Ca}^{2+}]_R$	the concentration of free Ca^{2+} in the cytosol at rest
$[\text{CaATP}]$	the concentration of Ca^{2+} bound to ATP
$[\text{CaD}]$	the concentration of Ca^{2+} bound to an indicator dye
$[\text{CaParv}]$	the concentration of Ca^{2+} bound to parvalbumin
$[\text{CaPump}]$	the concentration of Ca^{2+} bound to the SR Ca^{2+} pump
$[\text{CaTrop}]$	the concentration of Ca^{2+} bound to the regulatory sites on troponin
$[\text{Ca}_T]$	the concentration of total Ca^{2+}
D_{app}	the apparent diffusion coefficient of an indicator in the cytosol
$[D]$	the concentration of metal-free indicator
$[D_T]$	the total concentration of an indicator

f_{CaD}	the fraction of an indicator in the Ca^{2+} -bound form
f_{MgD}	the fraction of an indicator in the Mg^{2+} -bound form
F_R	indicator fluorescence intensity in the cytosol at rest
$FDHM$	the full-duration at half-maximum of a time-varying waveform

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Fig. 1.

Schematic of a portion of a frog skeletal muscle fibre. The arrows indicate the intracellular movement of Ca^{2+} during excitation-contraction coupling: release from the SR at the triadic junction, binding and diffusion within the myoplasm, and reuptake into the SR. $[\text{CaTrop}]$, $[\text{CaATP}]$, $[\text{CaParv}]$, and $[\text{CaPump}]$ denote the concentrations of Ca^{2+} bound to the major myoplasmic Ca^{2+} buffers (troponin, ATP, parvalbumin, and the SR Ca^{2+} pump). $[\text{CaD}]$ denotes the concentration of Ca^{2+} bound to an indicator dye introduced into myoplasm to measure free $[\text{Ca}^{2+}]$.

Fig. 2.

In vitro absorbance spectra of furaptra (A and C) and mag-fluo-4 (B and D). A) Squares: absolute spectra measured in zero and 10 mM Ca^{2+} ; curves: best-fitted spectra for zero and saturating Ca^{2+} based on these and other data (not shown). 30 μM furaptra; pH 7.0; 18 °C. B) Squares: absolute spectra measured in 0 and 0.24 mM $[\text{Ca}^{2+}]$; curves: best fitted spectra for 0 and saturating Ca^{2+} based on these and other data. 9 μM mag-fluo-4; pH 7.0; 21°C. C) and D) Difference spectra due to Ca^{2+} binding from A and B, respectively. All spectra were measured in a buffered KCl solution (0.15 M ionic strength); the cuvette path length was 1 cm.

Fig. 3.

Schematic view of one-fourth of a half sarcomere of one myofibril showing the arrangement of the 18 equal-volume compartments (6 longitudinal $\times$ 3 radial) in the multi-compartment model of the myoplasm used for frog fibres (Baylor and Hollingworth, 1998). SR Ca^{2+} release enters the half sarcomere in the outermost compartment nearest the z-line (large downward arrow). Ca^{2+} pump activity occurs within all compartments in the outer row (small upward arrows); troponin is restricted to the nine compartments located within $1\ \mu\text{m}$ of the z-line (the region containing thin filaments, average length $\sim 1\ \mu\text{m}$). The other buffers (ATP, parvalbumin, furaptra) are diffusible and therefore have access to all compartments. Because the buffer concentrations in Table 3 are average values for the entire myoplasmic volume, the actual concentrations of troponin and Ca^{2+} pump molecules in the relevant compartments are 2.0 and 3.0, respectively, times the values listed in Table 3.

Fig. 4.

Reaction schemes for ATP (A), parvalbumin (B), troponin (C), the SR Ca^{2+} pump (D), and furaptra (= Dye; E). Table 3 gives the total reactant concentrations and Table 4 the reaction rate constants. At the resting values of free $[\text{Ca}^{2+}]$, free $[\text{Mg}^{2+}]$, and pH in Table 3, the fractional occupancies of the different states of the various buffers at rest are: A) ATP (1.000), CaATP (0.000); B) Parv (0.062), CaParv (0.258), MgParv (0.680); C) Trop (0.993), CaTrop (0.006), Ca_2 Trop (.001); D) E (0.006), CaE (0.006), Ca_2 E (0.004), MgE (0.123), Mg_2 E (0.123), HE (0.062), H_2 E (0.615), H_3 E (0.062), H_4 E (0.001); E) Dye (0.451), CaDye (0.000), PrDye (0.548), CaPrDye (0.000). The percentage of furaptra in the protein-bound form at rest, 54.8%, is consistent with that estimated from furaptra's apparent myoplasmic diffusion coefficient, 54% (Table 2).

Fig. 5. Simulations of spatially-averaged Δf_{CaD} and $\Delta[\text{Ca}^{2+}]$ with the compartment model of Figs. 3–4 at three different values of indicator $K_{\text{D,Ca}}$: 1, 50 and 1,000 μM . A) The labeled traces show Δf_{CaD} for $K_{\text{D,Ca}} = 1$ and 50 μM ; the unlabeled trace shows Δf_{CaD} for $K_{\text{D,Ca}} = 1,000$ μM . $K_{\text{D,Ca}}$ here refers to the reaction of Ca^{2+} with protein-free indicator (cf. Fig. 4E); $K_{\text{D,Ca}}$ for protein-bound indicator is, in all cases, 2.14 times larger (cf. part E of Table 4). B) The continuous traces show the traces in A) after normalization to the same peak amplitude; the dashed trace is normalized $\Delta[\text{Ca}^{2+}]$, which is identical in the three simulations since $[\text{D}_\text{T}]$ was set to 1 μM (see text). The values of the peak and FDHM of $\Delta[\text{Ca}^{2+}]$ are 17.7 μM and 5.1 ms, respectively; the corresponding values for Δf_{CaD} are 0.702 and 61.6 ms, 0.140 and 8.2 ms, and 0.0117 and 5.3 ms ($K_{\text{D,Ca}}$'s of 1, 50 and 1000 μM , respectively). The Ca^{2+} release flux in the simulations was selected so that the amplitude and FDHM of the simulated Δf_{CaD} signal with $K_{\text{D,Ca}} = 50$ μM would roughly match that observed with fura-2 in a frog twitch fibre stimulated by an action potential. The equation for this flux (in $\mu\text{M}/\text{ms}$, referred to the volume of the half-sarcomere) is: $\text{Ca}^{2+} \text{ flux}(t) = 0$ if $t < 1.4$ else $= 17,642 \cdot [1 - \exp(-(t-1.4)/\tau_1)]^L \cdot \exp(-(t-1.4)/\tau_2) + 18.1 \cdot [1 - \exp(-(t-1.4)/\tau_1)]^L \cdot \exp(-(t-1.4)/\tau_3)$, where t is in ms, L is 5, and τ_1 , τ_2 , and τ_3 are 1.5, 0.6 and 6 ms, respectively. With this flux, $\Delta[\text{Ca}_\text{T}]$ (the total amount of released Ca^{2+}) is 365 μM , the peak rate of release is 151 $\mu\text{M}/\text{ms}$, the time of peak release is 3.1 ms, and the FDHM of release is 1.9 ms.

Fig. 6.

Simultaneous comparison of the ΔA signal from PDAA and the ΔF signal from furaptra in a frog twitch fibre stimulated to give an action potential at zero time (16 °C). The values of peak and FDHM of signals, after conversion to Δf_{CaD} units (Konishi et al., 1991), are 0.0096 and 6.3 ms (PDAA) and 0.119 and 9.1 ms (furaptra). The PDAA signal was obtained as a difference absorbance signal measured with wide-band filters centered at wavelengths 542 and 486 nm; the furaptra fluorescence signal was measured with a 420 ± 15 nm excitation filter and broad-band (490–590 nm) emission filter. Indicator concentrations were 1.25 mM (PDAA) and 0.26 mM (furaptra). Sarcomere length, 4.0 μm ; fibre diameter, 90 μm .

Fig. 7.

Comparisons of normalized $\Delta F/F_R$ signals measured simultaneously with mag-fluo-4 and furaptra in frog twitch fibres (16 °C). A) Responses to a single action potential. The values of peak and FDHM are 1.53 and 10.4 ms (mag-fluo-4) and -0.158 and 10.8 ms (furaptra). Sarcomere length, 3.6 μm ; fibre diameter, 54 μm ; indicator concentrations, 16 μM (mag-fluo-4) and 104 μM (furaptra). B) Responses to a single action potential and a 100-Hz train of five action potentials. The values of peak and FDHM elicited by the single action potential are 1.69 and 7.7 ms (mag-fluo-4) and -0.109 and 7.8 ms (furaptra). Sarcomere length, 3.5 μm ; fibre diameter, 100 μm ; indicator concentrations, 39 μM (mag-fluo-4) and 24 μM (furaptra). In part A, the standard deviation of the baseline noise is smaller for the furaptra trace than for the mag-fluo-4 trace; the reverse is true in part B. The fluorescence signals were measured as described by Hollingworth et al. (2009); 5 μM BTS was present in Ringer's in both experiments.

Fig. 8.

Comparisons of normalized $\Delta F/F_R$ signals measured simultaneously in frog fibres stimulated by an action potential (16 °C). A) Calcium-green-5N and furaptra. Peak and FDHM values are 0.96 and 36.8 ms (calcium-green-5N) and -0.132 and 10.2 ms (furaptra). Sarcomere length, 3.8 μm ; fibre diameter, 101 μm . B) OGB-5N and furaptra. Peak and FDHM values are 1.38 and 13.3 ms (OGB-5N) and -0.101 and 9.7 ms (furaptra). Sarcomere length, 3.3 μm ; fibre diameter, 93 μm . Signals were measured as described by Zhao et al. (1996) (part A) and Hollingworth et al. (2009) (part B); in B, the Ringer's contained 5 μM BTS.

Fig. 9.

Δf_{CaD} and tension responses averaged from four mouse fast-twitch fibres injected with furaptra and stimulated by a single action potential (A) and a 5-shock 67-Hz train of action potentials (B). Δf_{CaD} waveforms were measured at long sarcomere lengths at 16 °C as described by Hollingworth et al. (1996). The scale for the tension traces is relative to the maximum value in the 5-shock train. Because BTS was not used in these experiments, the Δf_{CaD} waveforms may be slightly contaminated with a movement artifact.

Fig. 10.

Comparison of measured and simulated spatially-averaged waveforms in mouse fast-twitch fibres. The upper traces show furaptra Δf_{CaD} measurements (noisy traces, from parts A and B of Fig. 9) and simulations (noise-free traces, calculated with the multi-compartment model as described in the text). The lower traces show the SR Ca^{2+} release waveforms used in the simulations.

Fig. 11.

Comparison of spatially-averaged $\Delta[\text{Ca}^{2+}]$ traces from the measurements and simulations of Figs. 9–10. The noisy traces were calculated from the measured Δf_{CaD} traces using equation 2 with $K_{\text{D,Ca}} = 96 \mu\text{M}$; the noise-free traces are from the simulations and are thought to more accurately reflect the true spatially-averaged $\Delta[\text{Ca}^{2+}]$. In A, the values of peak and FDHM of the $\Delta[\text{Ca}^{2+}]$ trace calculated with equation 2 are 17.5 μM and 4.6 ms, respectively; the corresponding values for the simulated $\Delta[\text{Ca}^{2+}]$ trace are 16.1 μM and 3.7 ms.

Fig. 12.

Extremes of modeled concentration changes in the single action potential simulation of Fig. 10. In each panel, the continuous traces show the largest and smallest concentration change among all compartments in the model for the indicated variables: $\Delta[\text{Ca}^{2+}]$; $\Delta[\text{CaParv}]$; $\Delta[\text{CaPump}]$; and $\Delta[\text{CaTrop}]$. The dashed traces show the spatially-averaged change for each variable. The increase in $\Delta[\text{CaParv}]$ occurs in two relatively distinct phases. The faster phase is due to Ca^{2+} binding to the Parv sites that are metal-free at rest (0.062 of the total; see legend of Fig. 4); the slower phase occurs as the Parv sites that have Mg^{2+} bound at rest (0.680 of the total) exchange Mg^{2+} for Ca^{2+} .

Table 1

In vitro properties of sixteen low-affinity Ca^{2+} indicators used in skeletal muscle

Indicator	$K_{D,\text{Ca}}$ (μM)	$K_{D,\text{Mg}}$ (μM)	$K_{D,\text{Mg}/D,\text{Ca}}$	λ_1 (nm)	λ_2 (nm)
A. Purpurate					
DMPDAA	780	-	-	515–525	555–585
PDAA	870–950	-	-	475–500	530–550
TMX	1940–2990	-	-	475–500	540–580
B. Tri-carboxylate					
Furaptra	44	5,300	120	390–430	>470
Mag-fura-5	31	4,300	139	390–430	>470
Mag-fura-red	55	9,300	169	465–495	>510
Mag-indo-1	29	7,600	262	390–430	>470
Mag-fluo-4	70	6,500	93	450–490	>510
Magnesium-orange	43	8,200	190	510–540	>590
C. Tetra-carboxylate					
BTC	26	-	-	390–430	>470
Calcium-green-5N	63–85	-	-	465–490	>510
Calcium-orange-5N	53–55	-	-	510–540	>590
OGB-5N	48	-	-	450–490	>510
Fluo-5N	90	-	-	450–490	>510
Fura-5N	47	-	-	390–430	>470
Rhod-5N	320	-	-	510–560	>575

$K_{D,\text{Ca}}$ and $K_{D,\text{Mg}}$ were measured in 0.10 – 0.15 M KCl, pH = 7.0 – 7.2, 16–22°C (Cifuentes et al., 1995; Hirota et al., 1989; Hollingworth et al., 2009; Maylie et al., 1987a; Raju et al., 1989; Vergara and Escobar, 1993; Zhao et al., 1996). λ_1 and λ_2 give the wavelengths that have been used most frequently to measure $\Delta[\text{Ca}^{2+}]$ in muscle fibres. In the case of the purpurate indicators, these are absorbance wavelengths; in the case of the carboxylate indicators, these are fluorescence excitation and emission wavelengths, respectively. Abbreviations: DMPDAA, dimethylpurpurate-3,3'diacetic acid; PDAA, purpurate-3,3'diacetic acid; TMX, tetramethylurexide; OGB-5N, Oregon-green bapta 488-5N.

Table 2

Comparison of results with low-affinity Ca^{2+} indicators in frog skeletal muscle fibres (16–18°C)

Indicator	D_{app} ($10^{-7} \text{ cm}^2 \text{ s}^{-1}$)	% binding	peak $\Delta\text{F}/\text{F}_R$	time of peak (ms)	FDHM (ms)
A. Purpurate					
DMPDAA*	10.8*	27*	(0.026*)	-	9.0*
PDAA	9.9	19*, 34	(0.018*), (0.011)	6.5	6.9*, 8.7
TMX	9.8	27*, 47	(0.006#), (0.005)	5.5	4.5#, 5.5
B. Tri-carboxylate					
Furaptra	7.3	54	(0.15)	5.2	10.6
Mag-fura-5	7.8	57	-0.132	5.2	10.6
Mag-fura-red	2.9	82	(signal waveform is biphasic)		
Mag-indo-1	4.4	73	-0.114	5.2	11.6
Mag-fluo-4	4.0	74	1.61	5.3	9.1
Magnesium-orange	2.3	84	0.145	5.0	12.5
C. Tetra-carboxylate					
BTC	0.7	95	0.144	6.3	16.2
Calcium-green-5N	1.2	91	0.835	6.3	36.7
Calcium-orange-5N	1.4	89	0.260	6.2	16.8
OGB-5N	2.7	80	1.16	6.0	16.6
Fluo-5N	-	-	1.88	6.3	19.6
Fura-5N	3.7	75	-0.076	5.8	10.9
Rhod-5N	1.9	86	5.4	7.2	14.5

Results in cut fibres at 18 °C are denoted by # (Maylie et al., 1987a) and at 16 °C by * (Hirota et al., 1989). All other results are in intact fibres at 16 °C (Hollingworth et al., 2009; Konishi and Baylor, 1991; Konishi et al., 1991; Zhao et al., 1996). The last 3 columns give average values of the spatially-averaged indicator response elicited by an action potential. A value in parentheses gives peak $\Delta\text{F}/\text{F}_R$; a value not in parenthesis gives peak $\Delta\text{F}/\text{F}_R$ measured with the excitation and emission wavelengths listed in Table 1. The values for Rhod-5N are for the time period 5–10 min after injection (Hollingworth et al., 2009).

Table 3

Modeled concentrations and diffusion coefficients in frog twitch fibres and mouse fast-twitch fibres (16 °C)

Constituent	Concentration (μM)	Concentration of binding-sites (μM)	Diffusion coefficient ($10^{-6} \text{ cm}^2 \text{ s}^{-1}$)
free $[\text{Ca}^{2+}]$	0.050 (resting)	-	3
free $[\text{Mg}^{2+}]$	1,000 (resting)	-	-
troponin	120	240 (Ca^{2+} regulatory sites)	0
SR Ca^{2+} pump	120	240 (Ca^{2+} transport sites)	0
parvalbumin	750	1500 ($\text{Ca}^{2+}/\text{Mg}^{2+}$ sites)	0.15
ATP	8,000	8000 ($\text{Ca}^{2+}/\text{Mg}^{2+}$ sites)	1.4
fura-2	100	100 ($\text{Ca}^{2+}/\text{Mg}^{2+}$ sites)	1.59
protein	500	500 (fura-2 sites)	0

Concentrations are spatially-averaged and referred to the myoplasmic water volume; except for free $[\text{Ca}^{2+}]$ and free $[\text{Mg}^{2+}]$, total concentrations are given. The concentrations of fura-2 (= dye) and protein (= Pr) apply to the standard model of Fig. 4E and Table 4.E. Free $[\text{Mg}^{2+}]$ is assumed to be constant during activity; pH is assumed to be 7 and constant. The diffusion coefficients of ATP and fura-2 apply to both the Ca^{2+} -free and Ca^{2+} -bound forms; the diffusion coefficient for parvalbumin applies to the Ca^{2+} -bound, Mg^{2+} -bound, and metal-free forms. Troponin, the SR Ca^{2+} pump, and protein-bound fura-2 are considered to be non-diffusible. See Baylor and Hollingworth (2007) and Hollingworth et al. (2006) for further information.

Table 4

Rate constants for the reactions shown in Fig. 4 (16 °C)

Reaction	Forward Rate (k_{on})	Reverse Rate (k_{off})	Ratio
A.			
$\text{Ca}^{2+} + \text{ATP} \rightleftharpoons \text{CaATP}$	$*0.1364 \times 10^8 \text{M}^{-1}\text{s}^{-1}$	$30,000 \text{s}^{-1}$	$2,200 \mu\text{M}$
B.			
$\text{Ca}^{2+} + \text{Parv} \rightleftharpoons \text{CaParv}$	$0.417 \times 10^8 \text{M}^{-1}\text{s}^{-1}$	0.5s^{-1}	$0.012 \mu\text{M}$
$\text{Mg}^{2+} + \text{Parv} \rightleftharpoons \text{MgParv}$	$0.33 \times 10^5 \text{M}^{-1}\text{s}^{-1}$	3s^{-1}	$91.0 \mu\text{M}$
C.			
$\text{Ca}^{2+} + \text{Trop} \rightleftharpoons \text{CaTrop}$	$1.77 \times 10^8 \text{M}^{-1}\text{s}^{-1}$	$1,544 \text{s}^{-1}$	$8.72 \mu\text{M}$
$\text{Ca}^{2+} + \text{CaTrop} \rightleftharpoons \text{Ca}_2 \text{Trop}$	$0.885 \times 10^8 \text{M}^{-1}\text{s}^{-1}$	17.1s^{-1}	$0.194 \mu\text{M}$
D.			
$\text{Ca}^{2+} + \text{E} \rightleftharpoons \text{CaE}$	$1.74 \times 10^8 \text{M}^{-1}\text{s}^{-1}$	6.97s^{-1}	$0.04 \mu\text{M}$
$\text{Ca}^{2+} + \text{CaE} \rightleftharpoons \text{Ca}_2\text{E}$	$1.74 \times 10^8 \text{M}^{-1}\text{s}^{-1}$	8.71s^{-1}	$0.05 \mu\text{M}$
$\text{Mg}^{2+} + \text{E} \rightleftharpoons \text{MgE}$	$8.71 \times 10^4 \text{M}^{-1}\text{s}^{-1}$	4.36s^{-1}	$50 \mu\text{M}$
$\text{Mg}^{2+} + \text{MgE} \rightleftharpoons \text{Mg}_2\text{E}$	$8.71 \times 10^4 \text{M}^{-1}\text{s}^{-1}$	87.1s^{-1}	$1,000 \mu\text{M}$
$\text{H}^+ + \text{E} \rightleftharpoons \text{HE}$	instantaneous	instantaneous	(pK=8)
$\text{H}^+ + \text{HE} \rightleftharpoons \text{H}_2\text{E}$	instantaneous	instantaneous	(pK=8)
$\text{H}^+ + \text{H}_2\text{E} \rightleftharpoons \text{H}_3\text{E}$	instantaneous	instantaneous	(pK=6)
$\text{H}^+ + \text{H}_3\text{E} \rightleftharpoons \text{H}_4\text{E}$	instantaneous	instantaneous	(pK=5)
$\text{Ca}_2\text{E} \rightleftharpoons \text{E} + (2 \text{Ca}^{2+})$	3.48s^{-1}	0	0
E.			
$\text{Ca}^{2+} + \text{Dye} \rightleftharpoons \text{CaDye}$	$2.33 \times 10^8 \text{M}^{-1}\text{s}^{-1}$	$11,416 \text{s}^{-1}$	$49 \mu\text{M}$
$\text{Pr} + \text{Dye} \rightleftharpoons \text{PrDye}$	$0.100 \times 10^8 \text{M}^{-1}\text{s}^{-1}$	$3,660 \text{s}^{-1}$	$366 \mu\text{M}$
$\text{Ca}^{2+} + \text{PrDye} \rightleftharpoons \text{CaPrDye}$	$0.466 \times 10^8 \text{M}^{-1}\text{s}^{-1}$	$4,909 \text{s}^{-1}$	$105 \mu\text{M}$
$\text{Pr} + \text{CaDye} \rightleftharpoons \text{CaPrDye}$	$0.100 \times 10^8 \text{M}^{-1}\text{s}^{-1}$	$7,869 \text{s}^{-1}$	$787 \mu\text{M}$

Letters A-E correspond to the reactions in panels A-E of Fig. 4, in which forward (column 2) and reverse (column 3) rate constants are denoted by k_{+i} and k_{-i} ($i = 1, 2$, etc.), respectively. Column 4 gives the dissociation constant ($= k_{-i}/k_{+i}$). In A, the asterisk in column 2 denotes an apparent rate constant at free $[\text{Mg}^{2+}] = 1 \text{ mM}$, $[\text{K}^+] = 0.15 \text{ M}$, $\text{pH} = 7$, and viscosity = 2 centipoise (Baylor and Hollingworth, 1998). In D, the dissociation constants of the reactions with H^+ are expressed as pKs (shown in parentheses); the parentheses on the right-hand side of the last reaction in column 1 denote the two Ca^{2+} ions transported by the pump to the lumen of the SR (see also Fig. 4D). In E, “Dye” denotes furaptra and “Pr” denotes protein sites capable of binding furaptra. Steady-state calculations with the scheme in E show that, with the protein concentration in Table 3, the relation between free $[\text{Ca}^{2+}]$ and the fraction of furaptra in the Ca^{2+} -bound form (in this case, $f_{\text{CaD}} = ([\text{CaDye}] + [\text{CaPrDye}]) / [\text{DT}]$) is very accurately described by an effective 1:1 binding equation with an apparent K_D of $70 \mu\text{M}$, the value estimated for frog myoplasm under steady-state conditions (Baylor and Hollingworth, 1998). However, when the peak of $\Delta[\text{Ca}^{2+}]$ elicited by an AP is calculated from Δf_{CaD} in a single-compartment calibration (equation (2)), a larger K_D , $96 \mu\text{M}$, is required (Baylor and Hollingworth, 1998).