Comparison of myoplasmic calcium movements during excitation-contraction coupling in frog twitch and mouse fast-twitch muscle fibers

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Single twitch fibers from frog leg muscles were isolated by dissection and micro-injected with furaptra, a rapidly responding fluorescent Ca\(^{2+}\) indicator. Indicator resting fluorescence (F\(_R\)) and the change evoked by an action potential (\(\Delta F\)) were measured at long sarcomere length (16°C); \(\Delta F/F_R\) was scaled to units of \(\Delta F_{Ca}\), the change in fraction of the indicator in the Ca\(^{2+}\)-bound form. \(\Delta F_{Ca}\) was simulated with a multicompartment model of the underlying myoplasmic Ca\(^{2+}\) movements, and the results were compared with previous measurements and analyses in mouse fast-twitch fibers. In frog fibers, sarcoplasmic reticulum (SR) Ca\(^{2+}\) release evoked by an action potential appears to be the sum of two components. The time course of the first component is similar to that of the entire Ca\(^{2+}\) release waveform in mouse fibers, whereas that of the second component is severalfold slower; the fractional release amounts are \(\sim 0.8\) (first component) and \(\sim 0.2\) (second component). Similar results were obtained in frog simulations with a modified model that permitted competition between Mg\(^{2+}\) and Ca\(^{2+}\) for occupancy of the regulatory sites on troponin. An anatomical basis for two release components in frog fibers is the presence of both junctional and parajunctional SR Ca\(^{2+}\) release channels (ryanodine receptors [RyRs]), whereas mouse fibers (usually) have only junctional RyRs. Also, frog fibers have two RyR isoforms, RyR\(_a\) and RyR\(_b\), whereas the mouse fibers (usually) have only one, RyR1. Our simulations suggest that the second release component in frog fibers functions to supply extra Ca\(^{2+}\) to activate troponin, which, in mouse fibers, is not needed because of the more favorable location of their triadic junctions (near the middle of the thin filament). We speculate that, in general, parajunctional RyRs permit increased myofilament activation in fibers whose triadic junctions are located at the z-line.

INTRODUCTION

In vertebrate skeletal muscle fibers, action potentials (APs) control contractile activity by eliciting rapid changes in the myoplasmic free Ca\(^{2+}\) concentration (\(\Delta [\text{Ca}^{2+}]\)). The amplitude and time course of the spatially averaged \(\Delta [\text{Ca}^{2+}]\) signal can be estimated with an appropriately chosen low-affinity Ca\(^{2+}\) indicator microinjected into the myoplasm (for review see Baylor and Hollingworth, 2011). From the indicator signal, kinetic modeling can be used to estimate the underlying Ca\(^{2+}\) movements, including (a) the release of Ca\(^{2+}\) from the SR, (b) the binding of Ca\(^{2+}\) to the major myoplasmic Ca\(^{2+}\) buffers (troponin, ATP, parvalbumin, and the SR Ca\(^{2+}\) pump), and (c) the reuptake of Ca\(^{2+}\) into the SR by the SR Ca\(^{2+}\) pumps.

The first detailed estimates of myoplasmic Ca\(^{2+}\) movements in skeletal muscle were carried out with single-compartment (spatially averaged) kinetic models (e.g., Robertson et al., 1981; Baylor et al., 1983; Melzer et al., 1987; Baylor and Hollingworth, 1988). A deficiency in single-compartment modeling is that it does not account for the substantial spatial gradients in \(\Delta [\text{Ca}^{2+}]\) that arise within the sarcomere because of the restricted locations of the SR Ca\(^{2+}\) release sites (Cannell and Allen, 1984; Escobar et al., 1994; Hollingworth et al., 2000; Gómez et al., 2006). More accurate estimates of the myoplasmic Ca\(^{2+}\) movements can be obtained with multicompartment (spatially resolved) modeling, in which Ca\(^{2+}\) release, Ca\(^{2+}\) pumping, and Ca\(^{2+}\) binding to the myoplasmic Ca\(^{2+}\) buffers are calculated for different locations within the sarcomere.

The first such multicompartment model analyzed Ca\(^{2+}\) transients measured in frog twitch fibers with aequorin during twitches and brief tetani (Cannell and Allen, 1984). Subsequently, multicompartment modeling was used to analyze frog Ca\(^{2+}\) transients measured with furaptra (Baylor and Hollingworth, 1998). Furaptra is a low-affinity rapidly responding Ca\(^{2+}\) indicator with some sensitivity to Mg\(^{2+}\) (Raju et al., 1989); the indicator’s myoplasmic fluorescence signal is thought to provide an accurate estimate of \(\Delta [\text{Ca}^{2+}]\) elicited by APs (Konishi et al., 1991; Baylor and Hollingworth, 2011). Thus, multicompartment modeling based on the furaptra Ca\(^{2+}\) signal likely gives reasonably accurate estimates of the amplitude and time course of SR Ca\(^{2+}\) release and the associated myoplasmic Ca\(^{2+}\) movements.
The aim of this study was to compare measurements and multicompartment simulations of the furaptra Ca\(^{2+}\) signal in frog twitch fibers with those previously carried out in mouse fast-twitch fibers of extensor digitorum longus (EDL) muscle (Baylor and Hollingworth, 2007, 2012). Comparisons of this type based on the use of identical techniques are of interest because of the finding that the SR Ca\(^{2+}\) release channels (also known as ryanodine receptors [RyRs]) differ in two important respects in adult skeletal fibers of amphibians and mammals. First, in mammals, a single RyR isoform, RyR1, is usually found, although there are small amounts of a second isoform, RyR3, in some fibers of specialized muscles such as diaphragm (e.g., Sorrentino, 2003). In contrast, in amphibians, two isoforms, denoted RyR\(\alpha\) and RyR\(\beta\), are found in approximately equal numbers in all major skeletal muscles (Olivares et al., 1991; Lai et al., 1992; Murayama and Ogawa, 1992). The amino acid composition of RyR\(\alpha\) and RyR\(\beta\) is analogous, respectively, to that of the mammalian RyR1 and RyR3 isoforms (Oyamada et al., 1994; Ottini et al., 1996).

A second difference between mammalian and amphibian RyRs concerns their anatomical arrangement at the triadic junctions, which are the sites of SR Ca\(^{2+}\) release during excitation–contraction (E-C) coupling. In mammals, the triadic junctions are located near the middle of the thin filament (Smith, 1966; Eisenberg, 1983; Brown et al., 1998), whereas, in amphibians, they are located near the z-line (e.g., Franzini-Armstrong, 1975). In addition, mammalian RyRs are found in a standard “junctional” configuration (Franzini-Armstrong and Nunzi, 1983; Block et al., 1988), which consists of a double-rowed array of RyRs in the region of the SR membrane located directly opposite a junctional region of transverse tubular (T-tubular) membrane (Fig. 1). The latter region contains its own double-rowed array of particles, the dihydropyridine receptors (DHPRs), which are the voltage sensors of E-C coupling (Ríos and Brum, 1987; Tanabe et al., 1988). The DHPRs are organized into groups of four, called “tetrads” (Franzini-Armstrong, 1984; Block et al., 1988; also see Fig. 1). The tetrads and junctional RyRs are present in a 1:2 stoichiometry, with one of every two RyRs positioned opposite a tetrad and the other positioned opposite a vacant space in the tetradic array (Franzini-Armstrong and Kish, 1995; Paolini et al., 2004). In amphibians, in contrast, only about half of all RyRs reside in this junctional configuration; the other half reside in a configuration termed “parajunctional” (Felder and Franzini-Armstrong, 2002). Parajunctional RyRs are found in double-rowed arrays that are offset \(\sim 40\) nm on either side of a junctional row (Fig. 1). The parajunctional arrays are incomplete, being, on average, only half-filled with RyRs, whereas the junctional arrays are fully filled. It is hypothesized that the junctional arrays contain only the RyR\(\alpha\) isoform and the parajunctional arrays only the RyR\(\beta\) isoform (Felder and Franzini-Armstrong, 2002). Overall, this arrangement would be consistent with the \(\sim 50:50\) numerical ratio of RyR\(\alpha\) to RyR\(\beta\) molecules reported in amphibians.

We report here that, in intact frog twitch fibers microinjected with furaptra and stimulated by an AP, the SR Ca\(^{2+}\) release waveform estimated with multicompartment modeling can be described as the sum of two kinetic components. The first component, with a fractional release amount of \(\sim 0.8\), has a time course that is similar to that of the entire SR Ca\(^{2+}\) release waveform estimated previously in fast-twitch and slow-twitch mouse fibers at the same temperature (16°C). The full duration at half maximum (FDHM) of these three release waveforms is \(\sim 2\) ms (frog twitch), \(\sim 1.6\) ms (mouse fast twitch; Baylor and Hollingworth, 2007), and \(\sim 1.7\) ms (mouse slow twitch; Hollingworth et al., 2012). Based on this similarity, it is reasonable to believe that the first release component in frog fibers and the entire release waveform in both types of mouse fibers are caused by a similar mechanism, namely, activation of junctional RyRs. The second release component in frog fibers, with a fractional release amount of \(\sim 0.2\), is substantially slower, with an estimated FDHM of \(\sim 9\) ms. We speculate that this release component is caused by Ca\(^{2+}\) release through parajunctional RyRs, the activation of which is secondary to that of junctional RyRs.

Our furaptra measurements in frog fibers were also analyzed under the hypothesis that Mg\(^{2+}\) competes with Ca\(^{2+}\) for occupation of the regulatory sites on troponin.

Figure 1. Cut-away schematic (Felder and Franzini-Armstrong, 2002) of essential E-C coupling molecules and membranes at the triadic junction of vertebrate skeletal muscle fibers. The diagram shows a junctional T-tubular membrane (light brown) flanked by two terminal-cisternal membranes of the sarcoplasmic reticulum (yellow). DHPR molecules (red) reside in the T-membrane; junctional RyRs (blue) and parajunctional RyRs (green) reside in the SR membrane. The parajunctional RyRs do not appear to contact the junctional RyRs (Felder and Franzini-Armstrong, 2002). See Introduction for further information, including the types of fibers in which parajunctional receptors are found. Copyright (2002) National Academy of Sciences, USA.
This possibility is supported by several studies in the literature (e.g., Godt and Lindley, 1982; Morimoto, 1991; Allen et al., 1992; Davis et al., 2002), but, to our knowledge, has not yet been considered in analyses of \( \Delta [Ca^{2+}] \) measurements in intact muscle cells. Estimates of SR Ca\(^{2+}\) release with our multicompartment modeling are not strongly affected by the assumption that Mg\(^{2+}\) binds to the troponin regulatory sites with a moderate affinity (Mg\(^{2+}\)-troponin dissociation constant = 2 mM; myoplasmic free [Mg\(^{2+}\)] = 1 mM). The main effects of including the myoplasm, was assumed to be 96 µM. Because large gradients in [Ca\(^{2+}\)] exist in myoplasm during activity, \( \Delta [Ca^{2+}] \) estimated with Eq. 2 likely involves some error (Hirota et al., 1989; Baylor and Hollingworth, 1998, 2007). A more accurate estimate of \( \Delta [Ca^{2+}] \) is thought to be obtained with multicompartment modeling (next section and Results).

**Materials and Methods**

**Ethical approval**
Animal protocols were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania.

**Experimental measurements**
The experiments were carried out as described previously (Konishi et al., 1991; Zhao et al., 1996). An intact single fiber from a frog leg muscle (semitendinosus or iliobibularis) was isolated by dissection, mounted on an optical bench apparatus, and bathed in a Ringer’s solution (16°C) that contained (in mM): 120 NaCl, 2.5 KCl, 1.8 CaCl\(_2\), and 5 PIPES, pH, 7.1. To minimize movement artifacts in the optical recordings, the fiber was stretched to a long sarcomere length (3.7 ± 0.1 µm; mean ± SEM, n = 7); in some experiments, 5 µM \( N \)-benzyl-p-toluene sulphphonamide (BTS) was included in Ringer’s solution to further reduce fiber movement. As shown previously (Cheung et al., 2002), BTS at this concentration is effective at suppressing the fiber’s contractile response without affecting the furaptra Ca\(^{2+}\) transient nor, by implication, the underlying myoplasmic Ca\(^{2+}\) movements. The fiber was pressure injected with the potassium-salt form of furaptra (Raju et al., 1989). The indicator fluorescence at rest (\( F_0 \)) and changes elicited by an AP (\( \Delta F \)) were measured from a 300-µm length of fiber that contained \( \approx 0.1 \) mM furaptra.

Although, in general, the diameter of frog twitch fibers is larger than that of mouse fast-twitch fibers from EDL muscle (the mouse fibers referenced in Results), an effort was made to match the diameters of the frog fibers to those of the mouse fibers. The diameter of each injected fiber was measured in one transverse dimension with a calibrated graticule. For the seven frog fibers of this study, the mean diameter was 53 ± 4 µm; the mean diameter for the eight fibers referenced in Results), an effort was made to match the diameters of each fiber's contractile response without affecting the furaptra, a three-state scheme for the reaction between Ca\(^{2+}\) and troponin, and a multistate scheme for the reaction between Ca\(^{2+}\) and the SR Ca\(^{2+}\) pump (Fig. 2 of Baylor and Hollingworth, 2007). Table 1 lists the concentrations of the myoplasmic Ca\(^{2+}\) buffers in the model and the diffusion coefficients of the mobile constituents.

A significant difference between the frog and mouse models is the site of SR Ca\(^{2+}\) release (large downward arrows in Fig. 2). In the frog model, Ca\(^{2+}\) enters the myoplasm in the compartment at the outer surface of the myofilbr next to the z-line, which approximates the location of the SR release sites in amphibian fibers (Franzini-Armstrong, 1975; Escobar et al., 1994; Tsugorka et al., 1995; Klein et al., 1996; Hollingworth et al., 2000). In the mouse model, the release compartment is offset to the middle of the troponin-containing region (middle of the thin filament), which approximates...
ESTIMATES OF: (A) THE LOCATION OF THE RELEASE SITES IN MAMMALIAN FIBERS (SMITH, 1966; EISENBERG, 1983; BROWN ET AL., 1998; GÓMEZ ET AL., 2006).

Activity in the model begins when an SR Ca\textsuperscript{2+} release flux enters the release compartment. This causes free Ca\textsuperscript{2+} in that compartment to rise, thus driving (a) complexation of Ca\textsuperscript{2+} with its buffers and (b) the diffusion of Ca\textsuperscript{2+} and the mobile Ca\textsuperscript{2+} buffers across the compartment’s boundaries. Analogous changes occur subsequently in the other compartments. The model thus yields compartment estimates of: (a) \(\Delta [\text{CaPump}]\), (b) the changes in the concentration of Ca\textsuperscript{2+} bound to the myoplasmic Ca\textsuperscript{2+} buffers (troponin, \(\Delta [\text{CaTrop}]\); ATP, \(\Delta [\text{CaATP}]\); parvalbumin, \(\Delta [\text{CaParv}]\); the SR Ca\textsuperscript{2+} pump, \(\Delta [\text{CaPump}]\); and furaptra, \(\Delta [\text{CaDye}]\)), (c) the myoplasmic diffusion of free Ca\textsuperscript{2+} and of Ca\textsuperscript{2+} bound to the mobile Ca\textsuperscript{2+} buffers (ATP, parvalbumin, and furaptra), and (d) the reuptake of Ca\textsuperscript{2+} into the SR by the SR Ca\textsuperscript{2+} pumps (\(\Delta [\text{CaPumped}]\)). Examples of such changes in the individual compartments of the model have been given previously (mouse model; Baylor and Hollingworth, 2007).

The rate at which Ca\textsuperscript{2+} is released from the SR is assumed to satisfy an empirical equation defined by rising and falling exponentials (Baylor and Hollingworth, 2007): Release rate \(r(t) = 0\) if \(t < T\), and is equal to

\[
r \times [1 - \exp(-(t - T)/\tau_r)] \times \exp(-(t - T)/\tau_f),
\]

if \(t \geq T\).

The time-shift parameter \(T\) (value, 1–2 ms) simulates the delay between the external shock that generates the AP and the onset of Ca\textsuperscript{2+} release; it is adjusted so that the rising phase of the simulated \(\Delta f_{\text{CaAD}}\) waveform matches that of the measured \(\Delta f_{\text{CaAD}}\) waveform. The amplitude parameter \(r\) is adjusted so that the peak of the simulated \(\Delta f_{\text{CaAD}}\) waveform matches that of the measurement. The success of the simulation is evaluated by comparing the overall time course of the simulated and measured \(\Delta f_{\text{CaAD}}\) waveforms.

SIMULATIONS THAT INCLUDE Mg\textsuperscript{2+} BINDING TO THE TROPOGIN REGULATORY SITES

To date, our simulations have assumed that the troponin regulatory sites bind Ca\textsuperscript{2+} specifically; i.e., without interference from Mg\textsuperscript{2+} (POTTER AND GERGELY, 1975; JOHNSON ET AL., 1981). However, several reports in the literature indicate that Mg\textsuperscript{2+}, at physiological concentrations, may bind significantly to the regulatory sites. The work of Davis ET AL. (2002) is particularly striking in this regard. These authors studied TnC from chicken skeletal muscle and demonstrated that the second Ca\textsuperscript{2+} regulatory site (EF-hand site II, countercurrent from the N terminus) binds Mg\textsuperscript{2+} with a dissociation constant \(K_{D,Mg} = 2–4\) mM, depending on measurement conditions; this value could be as low as 1–1.5 mM if cooperative binding of Ca\textsuperscript{2+} to the regulatory sites (Hill coefficient of 2) is considered in the analysis. The conclusion that Mg\textsuperscript{2+} can compete with Ca\textsuperscript{2+} for occupancy of the regulatory sites is strengthened by studies in skinned skeletal fibers that indicate that Mg\textsuperscript{2+}, at low millimolar concentrations, produces a right-shift in the tension–pCa curve (Godt and Lindley, 1982; ALLEN ET AL., 1992; DAVIS ET AL., 2002) and a right-shift in the Ca\textsuperscript{2+}-activated actomyosin ATPase curve (Morimoto, 1991).

Based on these findings, we have carried out some simulations (see Figs. 8, 10, and 11) that include competition between Mg\textsuperscript{2+} and Ca\textsuperscript{2+} for occupancy of the regulatory sites. For these simulations, the troponin reaction scheme shown in Fig. 3 was used, which is a simple extension of the scheme used previously for Ca\textsuperscript{2+}’s reaction with the troponin regulatory sites in our compartment model for frog twitch fibers (BAYLOR ET AL., 2002; HOLLINGWORTH ET AL., 2006) and mouse fast-twitch fibers (BAYLOR AND HOLLINGWORTH, 2007). The value chosen for \(K_{D,Mg} = k_{-3}/k_{+3}\) was 2 mM, which is in the range suggested by the work of Godt and Lindley (1982), ALLEN ET AL. (1992), and DAVIS ET AL. (2002). The on-rate constant \(k_{+3}\) was 6.0 \(\times 10^4\) M\textsuperscript{-1}s\textsuperscript{-1} (16°C), which is the mean of the on-rate constants in our model for the reaction of Mg\textsuperscript{2+} with the metal-free sites on parvalbumin (3.3 \(\times 10^4\) M\textsuperscript{-1}s\textsuperscript{-1}) and the Ca\textsuperscript{2+} pump (8.7 \(\times 10^4\) M\textsuperscript{-1}s\textsuperscript{-1}). The off-rate constant \(k_{-3}\) was 120 s\textsuperscript{-1}, calculated as the product of the dissociation constant and the on-rate constant.

**Statistics**

Student’s two-tailed \(t\) test was used to test for differences between population mean values, with the significance level set at \(P < 0.05\).

**RESULTS**

**Fiber measurements**

Fig. 4 shows an example of the tension response (lowest-trace) and the furaptra \(\Delta F/F_0\) signal (middle trace) recorded from a mouse fast-twitch fiber (A) and a frog twitch fiber (B) stimulated by a single AP (16°C). The top trace in each panel shows \(\Delta f_{\text{CaAD}}\) calculated from \(\Delta F/F_0\).
The FDHM values were 5.4 ± 0.5 ms and 10.1 ± 0.5 ms, respectively. The difference in peak values is not statistically significant (P > 0.05), whereas the difference in FDHMs is highly significant (P < 0.001). Although the mean diameters of the fibers selected for this comparison were significantly different (41 ± 2 µm for mouse and 53 ± 4 µm for frog; P = 0.009), the difference in FDHM does not appear to be attributable to the difference in fiber diameter. If the comparison of FDHMs is restricted to the fibers with closely similar diameters, namely the six largest mouse fibers (diameter, 44 ± 1 µm) and the three smallest frog fibers (diameter, 44 ± 2 µm), a similar difference in FDHM is observed, 5.7 ± 0.5 ms versus 9.2 ± 0.4 ms, respectively (P = 0.003). Thus, the difference in FDHM appears to reflect some consistent difference in the underlying myoplasmic Ca2+ movements in the two fiber types.

Single-compartment (spatially averaged) modeling

In Fig. 5, the lowermost trace in each panel shows the averaged \( \Delta f_{\text{CaD}} \) signal obtained from the fibers discussed in the preceding section (A, mouse, \( n = 8 \); B, frog, \( n = 7 \)). The upper traces in each panel show three spatially averaged estimates derived from the corresponding \( \Delta f_{\text{CaD}} \) trace: (a) \( \Delta [\text{Ca}^{2+}] \), which was calculated with Eq. 2; (b) the total amount of SR Ca2+ release (\( \Delta [\text{CaT}] \), with units referred to the myoplasmic water volume), which was estimated from \( \Delta [\text{Ca}^{2+}] \) and the single-compartment (spatially averaged) version of the myoplasmic Ca2+ model (e.g., as calculated with the method of Baylor et al., 1983; and Baylor and Hollingworth, 1988, 2003); and (c) the rate of SR Ca2+ release. In each panel, the release rate with noise was calculated from the corresponding \( \Delta [\text{CaT}] \)

![Figure 3. Four-state reaction scheme for the troponin regulatory sites in which Mg2+ competes with Ca2+ for binding to the metal-free state. The values of \( k_{+1}, k_{-1}, k_{+2}, \) and \( k_{-2} \) are 1.77 × 10^8 M\(^{-1}\)s\(^{-1}\), 1.544 s\(^{-1}\), 0.885 × 10^8 M\(^{-1}\)s\(^{-1}\), and 17.1 s\(^{-1}\), respectively (16°C; Baylor and Hollingworth, 2007). In the simulations without Mg2+ competition (Figs. 5–7, 9, 10 A, and 11 A), the troponin reaction included only the Mg2+-free states; with resting \( [\text{Ca}^{2+}] \) = 50 nM, the resting fractional occupancies are 0.995 (Trop), 0.006 (CaTrop), and 0.001 (Ca2Trop). In the simulations of Figs. 8, 10 B, and 11 B, the values of \( k_{+3} \) and \( k_{-3} \) were 6 × 10^6 M\(^{-1}\)s\(^{-1}\) and 120 s\(^{-1}\), respectively (KD,Mg = 2 mM). With resting \( [\text{Mg}^{2+}] \) = 1 mM, the resting fractional occupancies of the four states are 0.663 (Trop), 0.004 (CaTrop), 0.001 (Ca2Trop), and 0.332 (MgTrop). Table 1 gives the (spatially averaged) troponin concentration.

Results were compiled from several experiments like those in Fig. 4 in which contamination of the \( \Delta f_{\text{CaD}} \) measurements with movement artifacts was very small or negligible (mouse, \( n = 8 \); frog, \( n = 7 \)). In mice, the peak value of \( \Delta f_{\text{CaD}} \) was 0.157 ± 0.004 (mean ± SEM); in frogs it was 0.150 ± 0.012. The FDHM values were 5.4 ± 0.5 ms and 10.1 ± 0.5 ms, respectively. The difference in peak values is not statistically significant (P > 0.05), whereas the difference in FDHMs is highly significant (P < 0.001). Although the mean diameters of the fibers selected for this comparison were significantly different (41 ± 2 µm for mouse and 53 ± 4 µm for frog; P = 0.009), the difference in FDHM does not appear to be attributable to the difference in fiber diameter. If the comparison of FDHMs is restricted to the fibers with closely similar diameters, namely the six largest mouse fibers (diameter, 44 ± 1 µm) and the three smallest frog fibers (diameter, 44 ± 2 µm), a similar difference in FDHM is observed, 5.7 ± 0.5 ms versus 9.2 ± 0.4 ms, respectively (P = 0.003). Thus, the difference in FDHM appears to reflect some consistent difference in the underlying myoplasmic Ca2+ movements in the two fiber types.
More slowly than the simulated $\Delta f_{CaD}$. Because the concentrations and properties of the myoplasmic Ca$^{2+}$ buffers are thought to be similar in frog twitch fibers and mouse fast-twitch EDL fibers (e.g., Heizmann et al., 1982; Fink et al., 1986; Leberer and Pette, 1986; Godt and Maughan, 1988; Ecob-Prince and Leberer, 1989; Kushmerick et al., 1992), the discrepancy in Fig. 6 B suggests that the amount of SR Ca$^{2+}$ release in frog fibers is larger, and has a slower overall time course, in the measurements than in the simulations.

Fig. 7 A shows a comparison for frog fibers like that in Fig. 6 B except that the simulated Ca$^{2+}$ release rate is the sum of two components (broken lower traces), each of which satisfies the functional form of Eq. 3 (see legend). This release waveform has a slower final decay than that in Fig. 6 B and gives a somewhat larger total release (391 vs. 345 µM). Fig. 7 B shows the same comparison as that in Fig. 7 A but on a slower time base. The markedly improved overall agreement between the simulated and measured $\Delta f_{CaD}$ waveforms in Fig. 7 A compared with Fig. 6 B supports the conclusion that this two-component release waveform is a reasonable approximation of the actual release waveform in frog fibers. This result contrasts with that in mouse fast-twitch fibers (Fig. 6 A), where the single-component release function yielded a satisfactory multi-compartment simulation.

It is perhaps surprising that, in the spatially resolved modeling of Fig. 7 A, an excellent simulation of the frog $\Delta f_{CaD}$ waveform is obtained with a two-component Ca$^{2+}$ trace as $(d/dt)\Delta [Ca_T]$; the noise-free trace (Fig. 5, broken lines) is a mathematical approximation of this release rate that satisfies Eq. 3 (see figure legend). Each noise-free trace was used as the initial release waveform in the multicompartment modeling described in the next section. The use of the output of the single-compartment simulations is thought to be a reasonable starting point for the multicompartment simulations. The goal of the latter is to yield an internally consistent explanation of the measured $\Delta f_{CaD}$ signal in terms of the major underlying Ca movements taking place within a three-dimensional model of the fiber volume, such as is considered in our multicompartment simulations.

**Multicompartment (spatially resolved) modeling**

In Fig. 6, the upper traces in each panel show a comparison of simulated and measured $\Delta f_{CaD}$ waveforms (A, mouse; B, frog). Each simulated $\Delta f_{CaD}$ waveform (noise-free trace) was calculated with the multicompartment model (see Materials and methods) driven by a Ca$^{2+}$ release rate having the functional form of Eq. 3. The release waveforms (Fig. 6, lower traces) are similar to the noise-free traces (broken lines) in Fig. 5, with the amplitude of each waveform adjusted so that the peak of each simulated $\Delta f_{CaD}$ trace matched that of the corresponding measurement. In mouse fibers, the simulated and measured $\Delta f_{CaD}$ traces are in good agreement (Fig. 6 A), whereas in frog fibers they are not (Fig. 6 B). The main discrepancy in frog is that the measured $\Delta f_{CaD}$ decays more slowly than the simulated $\Delta f_{CaD}$. Because the concentrations and properties of the myoplasmic Ca$^{2+}$ buffers are thought to be similar in frog twitch fibers and mouse fast-twitch EDL fibers (e.g., Heizmann et al., 1982; Fink et al., 1986; Leberer and Pette, 1986; Godt and Maughan, 1988; Ecob-Prince and Leberer, 1989; Kushmerick et al., 1992), the discrepancy in Fig. 6 B suggests that the amount of SR Ca$^{2+}$ release in frog fibers is larger, and has a slower overall time course, in the measurements than in the simulations.

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release function even though the Ca\(^{2+}\) release waveform estimated in the spatially averaged modeling of Fig. 5 B has no discernible second component. This situation arises because of subtle differences inherent in the spatially averaged versus spatially resolved modeling approaches. For example, if the simulated \(\Delta f_{\text{CaD}}\) waveform from the spatially resolved model (Fig. 7 A) is used as the starting point in the spatially averaged model, a second release component, as expected, is not resolved.

Possible competition between Mg\(^{2+}\) and Ca\(^{2+}\) for the troponin regulatory sites
Several reports in the literature indicate that Mg\(^{2+}\) may compete with Ca\(^{2+}\) for occupancy of the troponin regulatory sites (see Introduction and Materials and methods), a possibility not considered in the previous modeling. To examine how such Mg\(^{2+}\) competition might impact the simulations, the reaction scheme shown in Fig. 3 was substituted for our usual troponin scheme; the concentration

Figure 6. Comparison of multicompartment simulations in mouse and frog fibers. In both fiber types, the simulated SR release waveform has the functional form of Eq. 3; the parameter values are similar to those in the corresponding parts of Fig. 5 except that the total release amounts and values of \(r_1\) and \(r_2\) are 349 \(\mu\)M, 1.3 ms, and 0.5 ms (A); and 345 \(\mu\)M, 1.75 ms, and 0.7 ms, respectively (B). With these values, the peak of each simulated \(\Delta f_{\text{CaD}}\) waveform matches that of the corresponding measurement. The FDHM of the simulated \(\Delta f_{\text{CaD}}\) waveform is 5.1 ms in A and 6.7 ms in B. The peak value, time of peak, and FDHM of the release waveforms are 205 \(\mu\)M/ms, 2.8 ms, and 1.6 ms (A); and 144 \(\mu\)M/ms, 2.9 ms, and 2.2 ms, respectively (B).

Figure 7. Comparison of simulated and measured frog \(\Delta f_{\text{CaD}}\) signals. (A) Comparison like that in Fig. 6 B except that the theoretical release function is the sum of two components (broken traces), each of which has the functional form of Eq. 3. The values of release amount, \(r_1\), and \(r_2\) are 335 \(\mu\)M, 1.5 ms, and 0.65 ms (first component); and 56 \(\mu\)M, 1.7 ms, and 7 ms, respectively (second component); the onset of the second component is assumed to lag that of the first component by 1 ms (\(T\) in Eq. 3 is 1 ms for the first component and 2 ms for the second). The peak value, time of peak, and FDHM of the composite release waveform are 157 \(\mu\)M/ms, 2.7 ms, and 2.0 ms. (B) Same traces as in A but on a fivefold slower time base.
Ca\(^{2+}\) reacted more rapidly with the regulatory sites. As noted previously (Baylor et al., 2002; Hollingworth et al., 2006), the reaction scheme between Ca\(^{2+}\) and troponin in our model is based on the cooperative binding of two Ca\(^{2+}\) ions to each troponin molecule (see Fig. 3 and legend). The limiting rate constant for the dissociation of Ca\(^{2+}\) from troponin (\(k_{-2} = 17.1\ s^{-1}; 16^\circ\text{C}\)) was chosen to be consistent with the experimental findings of Davis et al. (Davis, J.P., S.B. Tikunova, D.R. Swartz, and J.A. Rall. 2004. Biophysical Society Annual Meeting, Abstr. 1135). With this scheme and rate constants, good agreement was observed between the properties of simulated and measured Ca\(^{2+}\) sparks in frog intact fibers (measured with fluo-3; Baylor et al., 2002). This suggests that the Ca\(^{2+}\)–troponin reaction is reasonably well modeled, as troponin is the dominant myoplasmic Ca\(^{2+}\) buffer on a fast time scale and the simulated spark properties are sensitive to the troponin reaction rate constants. Nevertheless, because troponin’s reaction with Ca\(^{2+}\) is expected to have a significant influence in shaping the furaptra \(\Delta f_{\text{CaD}}\) waveform elicited by an AP and thus on the estimated amount of SR Ca\(^{2+}\) release, it was of interest to consider how the conclusions of the modeling would be affected if the actual Ca\(^{2+}\)–troponin rate constants are larger than the values assumed in the simulations of Figs. 5–8.

To investigate this question, a frog simulation like that in Fig. 7 was carried out with the values of the four reaction rate constants involving Ca\(^{2+}\) and troponin (see Fig. 3 and legend) increased twofold. This change does not affect the dissociation constants of the Ca\(^{2+}\)–troponin reactions and thus the steady-state value of \([\text{Ca}^{2+}]\) that gives half-occupancy of the troponin sites with Ca\(^{2+}\), of myoplasmic free \([\text{Mg}^{2+}]\) in the model was, as usual, 1 mM and constant. Fig. 8 shows results of a multicompartment frog simulation with this change. As in Fig. 7, a two-component release function was used (lowermost traces; see legend of Fig. 8), and good agreement was again observed between the simulated and measured \(\Delta f_{\text{CaD}}\) waveforms (middle traces). As expected, the spatially averaged change in the concentration of troponin molecules whose regulatory sites are both occupied with Ca\(^{2+}\) (Fig. 8, \(\Delta [\text{Ca}_2\text{Trop}]\), upper traces), which is the troponin state that is likely to be functionally important for activation of the fiber’s contractile response, has a smaller peak value in this simulation (99 \(\mu\text{M},\) continuous trace) than in the simulation of Fig. 7 (112 \(\mu\text{M},\) broken trace) and a later time of peak (20 vs. 15 ms).

The estimated amount of SR Ca\(^{2+}\) release is slightly smaller in the simulation of Fig. 8 than in Fig. 7 (374 vs. 391 \(\mu\text{M}\)) and the fractional amounts of the first and second release components differ modestly (0.76 and 0.24 in Fig. 8 vs. 0.86 and 0.14 in Fig. 7). Overall, the Ca\(^{2+}\) release waveform estimated in the modeling is not strongly affected by use of a troponin reaction with a moderate sensitivity to Mg\(^{2+}\).

Effects of faster reaction rates between Ca\(^{2+}\) and troponin

As noted in the preceding section, the overall time course with which Ca\(^{2+}\) binds to troponin is slower in the simulation in which Mg\(^{2+}\) is permitted to bind to the regulatory sites, and the estimated proportion of the released Ca\(^{2+}\) due to the second release component is increased somewhat. One would therefore expect that the fractional amount of release due to the second component would be reduced in a simulation in which Mg\(^{2+}\) increased twofold compared to the simulation in Fig. 7.

Figure 8. Results of a simulation like that in Fig. 7 except that the modeled troponin reaction included competition between Mg\(^{2+}\) and Ca\(^{2+}\) (see Fig. 3). In each panel (A, faster time base; B, slower time base), the superimposed traces at the top show simulated changes in the spatially averaged concentration of the doubly occupied Ca\(^{2+}\)–troponin state (rightmost state in Fig. 3), which were averaged over the nine troponin-containing compartments (see Fig. 2). The continuous trace is from this simulation; the broken trace is from the simulation of Fig. 7. For this simulation, the release amounts, \(r_1\), and \(r_2\) are 284 \(\mu\text{M},\) 1.5 ms, and 0.65 ms (first component); and 90 \(\mu\text{M},\) 1.7 ms, and 7 ms, respectively (second component). The peak value, time of peak, and FDHM of the composite release waveform are 135 \(\mu\text{M}/\text{ms},\) 2.7 ms, and 2.0 ms.
1.3 µM. In this simulation (not depicted), good agreement between the simulated and measured Δf_{CaD} traces was again observed (similar to that in Figs. 7 and 8). The estimated total concentration of released Ca^{2+}, 394 µM, is essentially identical to that in the simulation of Fig. 7, whereas the fractional amounts assigned to the first and second release components (0.88 and 0.12) differ slightly from that in the simulation of Fig. 7 (0.86 and 0.14). Overall, the Ca^{2+} release waveform estimated with the multicompartment model is not strongly affected by use of a troponin reaction scheme, with substantially increased rate constants between Ca^{2+} and the regulatory sites. Thus, simulations carried out under the assumption that Ca^{2+} binds to troponin more rapidly (this section) or more slowly (previous section) than is assumed in our standard model do not explain the observation of a slower decay of Δf_{CaD} in frog fibers compared with mouse EDL fibers. This result adds support to the interpretation that the slow decay of Δf_{CaD} in frog fibers is caused by an extra component of release that continues for some milliseconds after decline of the major portion of the release and not by an error in the choice of rate constants for the Ca^{2+}–troponin reaction.

**Effects of changes in other model parameters on the simulation of Δf_{CaD} in frog fibers**

Because parvalbumin, like troponin, binds a substantial amount of the released Ca^{2+} on the fast time scale of the measurements, simulations were also carried out with the multicompartement model to investigate whether the use of altered parameter values for parvalbumin in combination with the single-component Ca^{2+} release waveform would yield a satisfactory simulation of the frog Δf_{CaD} signal. The alterations considered included reductions in (a) the concentration of parvalbumin and (b) the rate constants for the reactions of Ca^{2+} and Mg^{2+} with parvalbumin. In both cases, parvalbumin should capture less Ca^{2+} than in the simulation with the standard parameter values, which should slow the decay of Δ[Ca^{2+}] from its peak and produce better agreement between the simulated and measured Δf_{CaD} waveforms. Results based on the first possibility are shown in the top panels of Fig. 9, in which the concentration of metal sites on parvalbumin was reduced twofold, from 1,500 to 750 µM (a change that is larger than reasonable). The time course of the single-component Ca^{2+} release waveform used to drive the simulation was identical to that in Fig. 6 B; as usual, the amplitude of the release flux was adjusted so that the peak of the simulated spatially averaged Δf_{CaD} waveform matched that of the measurement. Fig. 9 A reveals that, in comparison with the simulation of Fig. 6 B, there is, as expected, improved agreement with the measurement during the period 10–25 ms after stimulation; however, the later rising phase, time of peak, and early falling phase of the simulated waveform are in less-satisfactory agreement with the measurement than in the simulation of Fig. 6 B. In addition, Fig. 9 B reveals that the improved agreement in Fig. 9 A during the period 10–25 ms is not maintained on a time scale of 125 ms; during the period of 40–125 ms, a substantial discrepancy is observed between the simulated and the measured waveforms, which is greater than that seen in the simulations of Figs. 7 B and 8 B (both of which used a two-component release waveform). Results similar to those in Fig. 9 (A and B; not depicted) were observed if the parvalbumin site concentration was restored to its original value (1,500 µM), and the on- and off-rate constants for the reactions of Ca^{2+} and Mg^{2+} with parvalbumin were reduced twofold. Overall, these perturbations indicate that, with a single-component release function, simulations with the altered parameter choices for parvalbumin do not yield satisfactory agreement between the simulated and measured Δf_{CaD} waveforms.

Simulations with the single-component Ca^{2+} release function that included changes in several other modeling parameters were also explored, including changes in concentration of the SR Ca^{2+} pump molecules, changes in the rate constants with which Ca^{2+} and Mg^{2+} react with the Ca^{2+} pump, and changes in the concentration of ATP. When the concentration of Ca^{2+} pump molecules was set to half the normal value or when the reaction rate constants of the pump with Ca^{2+} and Mg^{2+} were reduced twofold, the simulated and measured Δf_{CaD} waveforms revealed substantial discrepancies (not depicted), similar to the discrepancies shown in Fig. 9 (A and B). When the ATP concentration was increased from 8 to 15 mM, better agreement between the simulated and measured Δf_{CaD} waveforms was observed (Fig. 9, bottom panels) than was seen with the other individual modeling changes that were investigated. However, even when this unreasonably large concentration of ATP was used in the model, the overall agreement between the simulated and measured Δf_{CaD} waveforms in Fig. 9 (C and D) is not as satisfactory as that in Figs. 7 and 8, where better agreement is observed in the later rising phase, time of peak, and early falling phase of the Δf_{CaD} waveforms (compare Fig. 9 C vs. Figs. 7 A and 8 A) and also in the late return toward baseline (40–125 ms; Fig. 9 D vs. Figs. 7 B and 8 B). Overall, the additional simulations described in this section indicate that the most straightforward modeling change that achieves a satisfactory simulation of the measured Δf_{CaD} signal in frog fibers is the addition of a small second component to the SR Ca^{2+} release waveform.

**Functional importance of the second release component in frog fibers**

As noted in Fig. 2, the site of SR Ca^{2+} release differs in the mouse and frog models, being positioned near the middle of the thin filament in the mouse model and near the z-line end of the thin filament in the frog model. The effect on the simulations due to this positional difference was considered previously in our mouse modeling carried

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out at a normal sarcomere length (2.4 µm; Baylor and Hollingworth, 2007). The principal effect noted was that, for the estimated Ca\textsuperscript{2+} release waveform elicited by an AP (lower trace in Fig. 6 A), the troponin molecules at the end of the thin filament closer to the m-line have a noticeably smaller Ca\textsuperscript{2+} occupancy in the case of the frog release location compared with the mouse location (mean peak of the Ca\textsubscript{Trop} state, 68% vs. 81% of maximum, respectively). This difference would be expected to have a significant effect on the amplitude of the twitch because this region of the thin filament is within reach of the myosin cross-bridges at most working sarcomere lengths. Thus, the mammalian release location has the functional advantage that, for a given release waveform, the released Ca\textsuperscript{2+} is more effective at activating the myofilaments. Because frog fibers do not have this advantage, it is necessary to release more Ca\textsuperscript{2+} to achieve the same degree of contractile activation.

Fig. 10 examines this point further by considering what compartment differences in \(\Delta[\text{Ca}^2+]\) and \(\Delta[\text{Ca}_\text{Trop}]\) occur if SR Ca\textsuperscript{2+} release in a frog fiber were to involve just the first release component of the two-component release waveform. The continuous traces in Fig. 10 (A and B) are taken from the simulations of Figs. 7 and 8, respectively (two-component release waveform), whereas the broken traces show the corresponding results if just the first release component in these simulations is used. The three \(\Delta[\text{Ca}^2+]\) traces of similar type (continuous or broken) show radially averaged changes in the free Ca\textsuperscript{2+} concentration at three longitudinal locations at increasing distances from the z-line (largest peak value, mean of the three radial compartments nearest the z-line; intermediate peak value, mean of the three radial compartments in the middle of the thin filament; smallest peak value, mean of the three radial compartments at the end of the thin filament closer to the m-line; see Fig. 2). The three \(\Delta[\text{Ca}_\text{Trop}]\) traces of similar type (broken or continuous) show the corresponding changes in the concentration of the Ca\textsubscript{Trop} state of troponin. In each comparison, the peak value of \(\Delta[\text{Ca}_\text{Trop}]\) averaged for the three troponin-containing compartments nearest the m-line is noticeably smaller in the simulation with one release component than with two. In Fig. 10 A, the peak values of these traces are 91 and 106 µM, respectively, which correspond to 76% and 89% of maximum; in Fig. 10 B, the corresponding values are 66 and 89 µM, which correspond to 56% and 75% of maximum. Overall, these percentage comparisons are similar to those mentioned in the preceding paragraph (68 vs. 81%), which apply if results for the mouse Ca\textsuperscript{2+} release waveform are compared at the frog and mouse release locations (simulation without Mg\textsuperscript{2+} competition for troponin; half-sarcomere length = 1.2 µm). These comparisons indicate that having both the first and second components of the Ca\textsuperscript{2+} release waveform at the frog release location (a) substantially increases Ca\textsuperscript{2+}’s occupancy of the troponin molecules nearest the m line, and (b) is functionally similar to having only the first release component at the mouse release location.

Simulations were also carried out with the short sarcomere model (half-sarcomere length = 1.2 µm) to examine the functional significance of the frog second release component in relation to the two different locations of SR Ca\textsuperscript{2+} release. The endpoint evaluated in these simulations was again the peak percentage of troponin molecules in the troponin compartments nearest the m-line that are in the Ca\textsubscript{Trop} state. If the mouse release waveform of Fig. 6 A was moved to the z-line end of the thin filament, these percentages were 68% without addition of the frog second release component of Fig. 7 and 85% with addition of this component. Conversely, if the first component of the frog release waveform of Fig. 7 was

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig9}
\caption{Results of frog simulations with a single-component Ca\textsuperscript{2+} release waveform and changes in parameter values for parvalbumin and ATP. (A) Comparison like that in Fig. 7 but with the concentration of parvalbumin sites reduced from 1,500 to 750 µM. The values of release amount, \(r_1\), and \(r_2\) are 317 µM, 1.75 ms, and 0.7 ms, respectively. The FDHM of the simulated \(\Delta f\textsubscript{CaD}\) waveform is 8.3 ms. (B) Same traces as in A but on a fivefold slower time base. (C and D). Comparison like that in Fig. 7 but with the concentration of ATP increased from 8 to 15 mM. The values of release amount, \(r_1\), and \(r_2\) are 399 µM, 1.7 ms, and 0.7 ms, respectively. The FDHM of the simulated \(\Delta f\textsubscript{CaD}\) waveform is 10.1 ms.}
\end{figure}
moved to the middle of the thin filament, these percentages were 76% without addition of the second release component and 87% with addition of the second component. These simulations at a physiological sarcomere length confirm the functional advantage of the second component of release in frog fibers, as inferred in the preceding paragraph in the simulations at long sarcomere length.

Measurements and simulations of $\Delta f_{\text{CaD}}$ in response to a brief high-frequency train of APs

Furaptra’s $\Delta f_{\text{CaD}}$ signal was also measured in several frog fibers that were stimulated to give 10 APs at 100 Hz. In five such experiments, the fibers were well immobilized and movement artifacts in the $\Delta F$ traces appeared to be small or negligible. The averaged $\Delta f_{\text{CaD}}$ signal from these fibers is shown in both panels of Fig. 11 (traces with noise at the top). This measurement was simulated with the two approaches used in Figs. 7 and 8; i.e., with a two-component release function both without and with competition between Mg$^{2+}$ and Ca$^{2+}$ for the troponin regulatory sites. The other traces in Fig. 11 summarize the results of these simulations. The lowermost trace in each part shows the release waveform used to drive the simulation. Each AP in the train was assumed to elicit a two-component release waveform whose functional form matched that in Figs. 7 and 8, respectively (see legend of Fig. 11); the amplitude of each AP-evoked release of Ca$^{2+}$ was adjusted in the usual way; i.e., so that each individual peak of $\Delta f_{\text{CaD}}$ in the simulated $\Delta f_{\text{CaD}}$ waveform matched the corresponding peak in the measurement. The second and third traces in each panel show two simulated $\Delta [\text{Ca}^{2+}]$ waveforms. The one of larger amplitude is $\Delta [\text{Ca}^{2+}]$ in the Ca$^{2+}$ release compartment (the outermost compartment nearest the z-line, Fig. 2), where, in response to the first AP, $\Delta [\text{Ca}^{2+}]$ reaches a peak of 90–100 µM and the subsequent peaks vary between ~45 and ~25 µM. The smaller $\Delta [\text{Ca}^{2+}]$ trace is spatially averaged $\Delta [\text{Ca}^{2+}]$; here, peak amplitudes during the train vary between ~14 and ~19 µM, and the shape of this waveform differs considerably from that of $\Delta [\text{Ca}^{2+}]$ in the release compartment. The top traces in each panel show that there is good agreement between the simulated and measured $\Delta f_{\text{CaD}}$ waveforms. In Fig. 11 A, the simulated amount of Ca$^{2+}$ release caused by the first AP is ~377 µM, and the subsequent releases, considered as a percentage of the first release, vary from ~22% to ~10%. The corresponding values in Fig. 11 B are 343 µM, 29%, and 11%, respectively. The large reductions in release that occur with the second and subsequent APs are likely caused by the process of Ca$^{2+}$ inactivation of Ca$^{2+}$ release, in which the $\Delta [\text{Ca}^{2+}]$ elicited by a prior AP feeds back in a negative fashion to inhibit the amount of Ca$^{2+}$ released by the later APs in the train (see Discussion).

**DISCUSSION**

In frog twitch fibers activated by an AP, the amplitude of the furaptra $\Delta f_{\text{CaD}}$ signal is similar to that in mouse fast-twitch EDL fibers but the decay of $\Delta f_{\text{CaD}}$ is slower. The conditions for measurement of the $\Delta f_{\text{CaD}}$ signals in the two fiber types were essentially identical, including the use of dissected intact fibers (single fibers for frog, small fiber bundles for mouse), the temperature (16°C), the mean sarcomere length of the fibers (3.7 ± 0.1 µm in both

Figure 10. Differences in $\Delta [\text{Ca}^{2+}]$ and $\Delta [\text{Ca}^{2+}]_{\text{Trop}}$ waveforms in frog simulations that included both components of the release function (continuous traces) and only the first component of the release function (broken traces). In A, the troponin reaction did not include Mg$^{2+}$ competition (simulated with the release function from Fig. 7); in B, the reaction in Fig. 3 was used (simulated with the release function from Fig. 8). Among any three traces of similar type (broken or continuous), the traces with the largest, intermediate, and smallest peak values are the averages, respectively, of the changes in the three radial compartments adjacent to the z-line, the three radial compartments in the middle of the thin filament, and the three radial compartments at the m-line end of the thin filament (compare with Fig. 2). The length of the half-sarcomere in the simulation was 2.0 µm (as in Figs. 6–9).
Comparisons with measurements and proposals in the literature

The mechanism of activation of RyRs is hypothesized to differ significantly in mammalian and amphibian fibers. In mammals, the junctional RyRs that are apposed by DHPR tetrads (see Introduction and Fig. 1) are thought to be under conformational control of the overlying DHPRs via a direct protein–protein interaction (Schneider and Chandler, 1973; Beam and Horowicz, 2004), a mechanism sometimes called voltage-induced Ca$^{2+}$ release (VICR). Exactly how control of the junctional RyRs that are not apposed by DHPR tetrads is achieved is an open question. Possibilities include: (a) Ca$^{2+}$-induced Ca$^{2+}$ release (CICR; e.g., Ríos and Pizarro, 1988; Stern et al., 1997), in which Ca$^{2+}$ ions released from RyRs apposed by tetrads reach the nonapposed RyRs by diffusion and activate them via a ligand interaction; and (b) coupled-gating (Marx et al., 1998), in which a conformational change in an activated RyR apposed by a tetrad activates an adjacent (nonapposed) RyR via a protein–protein interaction. In amphibians, the parajunctional RyRs appear to be too far removed from the junction to be activatable by either VICR or coupled gating, but, as proposed by Felder and Franzini-Armstrong (2002), studies, the mean diameter of the fibers (53 ± 4 µm for frog, n = 7; 41 ± 2 µm for mouse, n = 8), and the myoplasmic concentration of furaptra (~0.1 mM). Thus, we are confident that the comparison of the experimental properties of $\Delta f_{\text{CaD}}$ in the two fiber types is valid. Because the concentrations and properties of the myoplasmic Ca$^{2+}$ buffers appear to be similar in these fiber types (e.g., Heizmann et al., 1982; Fink et al., 1986; Leberer and Pette, 1986; Godt and Maughan, 1988; Ecob-Prince and Leberer, 1989; Kushnerick et al., 1992), the slower decay of $\Delta f_{\text{CaD}}$ in frog fibers suggests that the overall time course of Ca$^{2+}$ release is slower, and the release amount is larger, in frog than in mouse fibers. Multicompartment modeling reveals that the frog $\Delta f_{\text{CaD}}$ signal elicited by an AP is well simulated under the assumption that the Ca$^{2+}$ release waveform is the sum of two kinetic components, a larger and faster one (fractional release amount, ~0.8; FDHM, ~2 ms), and a smaller and slower one (fractional release amount, ~0.2; FDHM, ~9 ms). The FDHM of the faster component is similar to that of the entire SR Ca$^{2+}$ release waveform estimated previously in mouse fast-twitch fibers, 1.6 ms (Baylor and Hollingworth, 2007), and in mouse slow-twitch fibers, 1.7 ms (Hollingworth et al., 2012).

**Figure 11.** Comparison of simulated and measured frog $\Delta f_{\text{CaD}}$ signals elicited by 10 APs at 100 Hz. In A, the simulated troponin reaction did not include Mg$^{2+}$ competition; in B, the reaction in Fig. 3 was used. The lowermost trace in each panel shows the Ca$^{2+}$ release waveform used to drive the simulation. The next trace shows simulated $\Delta [\text{Ca}^{2+}]$ in the release compartment (see Fig. 2), and the third trace is the spatially averaged $\Delta [\text{Ca}^{2+}]$ (i.e., the average of the $\Delta [\text{Ca}^{2+}]$ waveforms in all 18 compartments of the model). The top traces compare the simulated and measured $\Delta f_{\text{CaD}}$ signals (noise-free and noisy traces, respectively). The measured $\Delta f_{\text{CaD}}$ signal (which is shown in both panels) was averaged from five fibers in which contamination of the fluorescence signal with movement artifacts was very small or negligible. The mean diameter and mean sarcomere length of these fibers were 54 ± 3 µm (mean ± SEM) and 3.7 ± 0.1 µm, respectively. The release functions for the simulations in A and B were based on the two-component release functions in Figs. 7 and 8, respectively. For convenience in the calculation, it was assumed that the onset of each subsequent release extinguished any residual release elicited by the preceding APs and that the relative amplitudes of the two release components remained unchanged. Although it is uncertain whether the relative amplitudes of the two release components remain unchanged with the second and subsequent releases, measurements in frog cut fibers indicate that virtually all of the Ca$^{2+}$ release channels (>90%) are subject to Ca$^{2+}$ inactivation of Ca$^{2+}$ release (Jong et al., 1995). The simulated release due to the first AP continued to 10.6 ms, the time at which the second release became significant. The second and subsequent releases were shifted in successive 10-ms increments and their amplitudes scaled down appropriately. In A, the amount of Ca$^{2+}$ released with the first AP is 377 µM and the amounts of the subsequent releases, expressed as a percentage of this amount, are 21.8, 17.5, 14.9, 12.6, 11.7, 10.4, 10.2, 9.5, and 10.0%, respectively. In B, the corresponding values are 345 µM and 29.2, 22.8, 16.4, 14.7, 12.7, 11.7, 10.8, 10.1, and 11.2%.
might be activated by CICR via the Ca\textsuperscript{2+} ions released, at least initially, from junctional RyRs. The presence of parajunctional RyRs in amphibian fibers is consistent with proposals in the literature that CICR is mechanistically significant in amphibian fibers (Rios and Pizarro, 1988; Jacquemond et al., 1991; Pizarro et al., 1992; O’Brien et al., 1995; Sutko and Airey, 1996; Klein et al., 1996; Pape and Carrier, 1998; González et al., 2000; Pape et al., 2002).

Our description of two release components in frog fibers and one component in mouse fibers is qualitatively consistent with the literature proposals that SR Ca\textsuperscript{2+} release in (adult) amphibian fibers depends on both VICR and CICR, whereas that in (adult) mammalian fibers is due primarily to VICR. It is also qualitatively consistent with the finding that amphibian RyRs are comprised of approximately equal amounts of two isoforms, RyR\textalpha and RyR\textbeta, whereas the RyRs in (most) mammalian fibers consist of one main isoform, RyR1 (see Introduction). An obvious interpretation of our results (Figs. 7, 8, and 11) is that the modeled first component of the frog release waveform reflects activity of junctional RyRs, perhaps exclusively activated by VICR, whereas the second component reflects activity of parajunctional RyRs, perhaps exclusively activated by CICR.

**Functional significance of the second release component**

As mentioned in Results (Fig. 10 and associated text), our simulations in frog fibers indicate that the peak change in concentration of the Ca\textsuperscript{2+}Trop state of the troponin molecules that are most distant from the z-line is substantially increased by the presence of the second component of release. Activation of these troponin molecules is likely to be functionally quite important at most working sarcomere lengths, and inclusion of the second release component in the frog model reveals a degree of troponin activation that is similar to that which occurs in the mouse model, which does not include a second release component and where the triadic junctions are located more centrally along the length of the thin filament. One may therefore speculate that two design strategies have evolved among adult vertebrate twitch fibers to achieve a high degree of troponin activation along the entire length of the thin filament. The second strategy is represented by most fibers of mammals as well as those of many reptiles (e.g., lizards and snakes; O’Brien et al., 1995), which have only one RyR isoform (namely, RyR1 or its equivalent) and may have only junctional RyRs. An exception to the z-line location of the triadic junctions in fish muscle occurs in the superfast fibers of the toadfish swim bladder muscle, whose triadic junctions are located near the middle of the thin filament. Interestingly, swim bladder fibers have only a single RyR isoform (O’Brien et al., 1993), lack parajunctional RyRs (Felder and Franzini-Armstrong, 2002), and have a brief Ca\textsuperscript{2+} release waveform (Harwood et al., 2011), i.e., they adhere to the second design strategy. In summary, we speculate that the (most common) function of the parajunctional RyRs in adult vertebrate fibers is to permit a modest increase in the amount of SR Ca\textsuperscript{2+} release with an AP when triads are located near the z-line; this compensates for the smaller activation of troponin that would otherwise occur given that the z-line location for Ca\textsuperscript{2+} release is inherently less favorable for complete activation of the myofilaments (Fig. 10 and associated text). In terms of the peak occupancy of troponin with Ca\textsuperscript{2+}, the benefit of this extra release would likely apply primarily to the activation of troponin in response to a single AP or a series of APs that do not occur in close succession, because, with a high-frequency stimulus, most of the regulatory sites on troponin would likely be bound with Ca\textsuperscript{2+} by the time of the second or third AP in the stimulus train. Even with a high-frequency stimulus, some benefit may arise from the second component in having a more synchronous activation of troponin along the length of the thin filament.

**Other experimental approaches and alternative interpretations**

Although we believe that the presence of a second release component is the most likely explanation of the slow decay of the furaptra \(\Delta F_{\text{GAD}}\) waveform that we have measured in frog fibers, it should be acknowledged that the evidence for this is indirect, as it relies on inferences based on a complex multicompartment kinetic model whose parameters are not all known with a high degree of certainty. In addition, the basic measurement of \(\Delta F_{\text{GAD}}\) is a spatially averaged one in which fluorescence is collected from a \(\sim300-\mu\text{m}\) fiber length and the full fiber width, the volume of which is many-fold larger than the simulation volume, which considers only a half-sarcomere of one myofibril. Counter-balancing these concerns, we note that (a) the basic structure of both frog twitch fibers and mouse fast-twitch fibers is a highly repetitive one that is reflected in the sarcomeric and myofibrillar features incorporated into the model, and (b) the measurement and simulation of the \(\Delta F_{\text{GAD}}\) signal in frog fibers revealed clear differences when compared with that in mouse fibers. Nevertheless, it would clearly be advantageous to have an experimental method that more directly assessed
the properties of the inferred Ca\(^{2+}\) release waveforms in the two fiber types. It does not appear that a straightforward use of spatially resolved imaging techniques to measure a Ca\(^{2+}\)-indicator signal in response to AP stimulation (e.g., as used by Escobar et al., 1994; Monck et al., 1994; Hollingworth et al., 2000; and Zoghi et al., 2000) would be useful in this regard. These measurements, as well as those in this study, monitor a signal that is directly related to \(\Delta[Ca^{2+}]\) rather than the Ca\(^{2+}\) release waveform per se (whose time course is quite different from that of \(\Delta[Ca^{2+}]\)). Indicator-dye measurements that more directly monitor the Ca\(^{2+}\) release waveform can be made if a large concentration of an exogenous high-affinity Ca\(^{2+}\) buffer is present in the myoplasm (e.g., Baylor and Hollingworth, 1988; Jong et al., 1995; Pape and Carrier, 1998); with this approach, however, any process dependent on CICR, as proposed for activation of the parajunctional Ca\(^{2+}\) release channels, would be expected to be suppressed. A pharmacological approach might provide a successful test for the presence of two kinetically distinguishable release components in fibers with both junctional and parajunctional receptors; for example, if a release-channel inhibitor were available that was selective for RyR\(\beta\) over RyR\(\alpha\) and did not affect other processes in the E-C coupling sequence. Unfortunately, we are not aware of the existence of such an inhibitor.

Finally, it should be pointed out that factors other than activation of parajunctional RyRs might contribute to the difference in the measured properties of the \(\Delta f_{CaD}\) signal in frog versus mouse fibers. For example, we cannot rule out the possibility that some difference in the AP or in the functioning of the DHPRs, including the amplitude and time course of muscle charge movement elicited by the AP, occurs in the two fiber types that might contribute to, or perhaps entirely account for, the difference in the \(\Delta f_{CaD}\) signals.

Inactivation of Ca\(^{2+}\) release
If CICR is the mechanism of activation of the parajunctional RyRs and/or the junctional RyRs that are not located directly opposite DHPR tetrads, the potential for a regenerative release of SR Ca\(^{2+}\) appears to exist in all skeletal fiber types. In this circumstance, it would be important to have some control capable of inhibiting Ca\(^{2+}\) release. The process of Ca\(^{2+}\) inactivation of Ca\(^{2+}\) release (Baylor et al., 1983; Schneider and Simon, 1988; Baylor and Hollingworth, 1988; Jong et al., 1995) appears capable of supplying this control. In frog cut fibers activated by a step depolarization from −90 mV to a positive potential, Ca\(^{2+}\) inactivation of Ca\(^{2+}\) release appears to rapidly inhibit >90% of the fiber’s Ca\(^{2+}\) release capability (Jong et al., 1995). Our experiments on frog intact fibers stimulated by a high-frequency train of APs (Fig. 11 and associated text) are generally consistent with these results and with those of Pape et al. (1993) on cut fibers stimulated by multiple APs. We estimate that, with a 100-Hz stimulus of a frog intact fiber at 16°C, the amount of Ca\(^{2+}\) released with the second AP in the train is 20–30% of that of the first release, and, by the 10th AP, the amount is ~10% of the first release (Fig. 11). It is likely that this strong suppression of release is due mainly to the process of Ca\(^{2+}\) inactivation (Pape et al., 1993; Jong et al., 1995). A rapid onset of Ca\(^{2+}\) inactivation of the release system, perhaps in combination with a resting inhibition of parajunctional RyRs due to a myoplasmic free Mg\(^{2+}\) concentration near 1 mM, may be the reason that the slower second component of the release waveform represents only ~20% of the total release (one AP; Figs. 7 and 8), whereas the parajunctional RyRs, whose secondary activation may underlie the slower component of release, are found in numbers approximately equal to that of the junctional RyRs.

Ca\(^{2+}\) inactivation of Ca\(^{2+}\) release also appears to be of major importance in the control of Ca\(^{2+}\) release in fibers that contain only junctional RyRs and have a single RyR isoform, including mammalian fast-twitch fibers (Hollingworth et al., 1996; Baylor and Hollingworth, 2007), mammalian slow-twitch fibers (Baylor and Hollingworth, 2003; Hollingworth et al., 2012), and toadfish superfiber fibers (Harwood et al., 2011). When these fibers are stimulated by a train of five APs at 67–83 Hz, the amount of release with the second and subsequent APs is estimated to be 26–15% of the first release in fast-twitch fibers, 30–13% in slow-twitch fibers, and 35–30% in superfiber fibers (16°C). Overall, this mechanism (a) prevents [Ca\(^{2+}\)] from rising to higher levels than is needed to activate the myofilaments, thereby reducing the expenditure of ATP for the resequstration of Ca\(^{2+}\); (b) helps avoid unnecessary delays in relaxation; and (c) limits the entry of potentially toxic amounts of Ca\(^{2+}\) into the mitochondria.

Possible competition between Mg\(^{2+}\) and Ca\(^{2+}\) for troponin
As mentioned in the Introduction, several reports in the literature suggest that Mg\(^{2+}\), at physiological levels, competes with Ca\(^{2+}\) for occupancy of the troponin regulatory sites. To our knowledge this possibility has not yet been considered in analyses of \(\Delta[Ca^{2+}]\) measurements in muscle cells. Interestingly, results with our multicompartiment modeling (Figs. 8, 10, and 11) are not strongly affected under the hypothesis that Mg\(^{2+}\) binds to the troponin regulatory sites with a moderate affinity (dissociation constant = 2 mM; myoplasmic free [Mg\(^{2+}\)] = 1 mM). In comparison with the noncompetitive troponin model, the time course of Ca\(^{2+}\) release in frog fibers estimated in these simulations is prolonged slightly due to a modest increase in the amount of the second release component (fractional amount, 0.24 vs. 0.15), the total amount of Ca\(^{2+}\) release is reduced by ~5%, the peak spatially averaged change in the Ca\(^{2+}\) occupancy of the troponin regulatory sites is reduced by ~12%, and the peak time of this occupancy is increased from 15 to 20 ms (16°C).
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