CYTOPLASMIC FREE CONCENTRATIONS OF Ca^{2+} IN SKELETAL MUSCLE CELLS

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1. INTRODUCTION

Because cytoplasmic free Ca^{2+} regulates muscle contraction (Ebashi and Endo, 1968), it is essential to obtain accurate information on the levels of cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$), as well as the time course and amplitude of $[Ca^{2+}]_i$ changes (Ca^{2+} transients), to study excitation-contraction coupling of muscle cells. Ca^{2+} transients in skeletal muscles have been measured with many different methodologies, such as optical indicators, bioluminescent proteins and Ca^{2+}-selective microelectrodes. Since each method has various advantages and disadvantages in the measurement of Ca^{2+} transients, significant controversy still remains concerning the quantities reported by different techniques. In this chapter, I will review developments of $[Ca^{2+}]_i$ measurements in skeletal muscle cells, and also briefly describe our experiments carried out to estimate $[Ca^{2+}]_i$ at rest and during steady state contraction in frog skeletal muscle fibers.

The first measurements of Ca^{2+} transients in skeletal muscle were reported by Jöbsis and O'Connor (1966) who introduced murexide into toads by intraperitoneal injection and observed changes in murexide absorbance in the sartorius muscle in response to electrical stimulation. Because murexide is a dye that undergoes absorbance changes upon Ca^{2+} binding, the authors attributed the optical signals to Ca^{2+} transients. However, their "Ca^{2+} transients" have been questioned, because of their very slow time course (time to peak 50-100 ms after stimulation) For example, Maylie et al. (1987b) used tetramethylmurexide, a dye structurally related to murexide, and showed very rapid changes in its absorbance with a time to peak of about 5 ms after stimulation.

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2. MEASUREMENTS OF Ca^{2+} TRANSIENTS

2.1. Bioluminescent proteins

Shimomura et al. (1962) extracted aequorin, a Ca^{2+} sensitive bioluminescent protein, from a jelly fish *Aequorea aequorea* and clarified fundamental properties of its luminescence. Ashley and Ridgway (1968) injected aequorin into striated muscles of a barnacle and simultaneously measured the membrane potential, Ca^{2+} transients and tension. This work clearly demonstrated the central role of cytoplasmic Ca^{2+} in excitation-contraction coupling. Properties of aequorin were further studied extensively by Blinks and his collaborators (1976, 1982), and aequorin was applied to frog skeletal muscle fibers [Rüdel and Taylor (1973), Blinks et al. (1978)] and also to cardiac and smooth muscles [Allen and Blinks (1978), Allen and Kurihara (1982), Morgan and Morgan (1982)]. In general, aequorin is a good choice to simultaneously measure Ca^{2+} transients and mechanical activities in skeletal, cardiac and smooth muscles. Recently, recombinant versions of aequorin can be inserted and expressed in target organelles (e.g., mitochondria) of cultured cells as a useful tool to measure Ca^{2+} concentrations in organelles.

2.2 Metallochromic dyes

Metallochromic dyes undergo absorbance changes upon Ca^{2+} binding. Among this family of dyes, arsenazao III and antipyrylazo III have been widely used. Miledi et al. (1977) injected arsenazao III into frog skeletal muscle fibers, and measured Ca^{2+} transients under voltage-clamp or following action potentials. Disadvantages of metallochromic dyes include complex stoichiometry of Ca^{2+}-dye binding; for example, arsenazao III binds Ca^{2+} with 1:1, 1:2, 2:1 and 2:2 stoichiometries (Dorogi and Neumann, 1981; Rios and Schneider 1981; Palade and Vergara, 1983). This makes it very difficult to calibrate the dye signals in terms of $[Ca^{2+}]_i$. For antipyrylazo III, the 1:2 Ca^{2+}:indicator complex appears to be predominantly formed at the concentrations of antipyrylazo III and Ca^{2+} normally encountered in the cytoplasm (Rios and Schneider, 1981; Hollingworth et al., 1987). The absorbance signal from the 1:2 Ca^{2+}:antipyrylazo III complex is considered to follow a rapid change in $[Ca^{2+}]_i$ during twitch response with a relatively small (1-2 ms) kinetic delay in frog skeletal muscle fibers (Baylor et al., 1985; Maylie et al., 1987a). On the other hand, the peak amplitude of the cytoplasmic Ca^{2+} transient calibrated from antipyrylazo III is 2-3 μM (Maylie et al., 1987a; Baylor and Hollingworth, 1988), which may be erroneously small, probably because ~75% of the cytoplasmic antipyrylazo III molecules appear to be bound to intracellular constituents, and the properties of the bound indicator molecules are likely to be different from those in salt solutions (Baylor et al., 1986). The calibration difficulties arising from the binding of an indicator to cellular constituents applies not only to metallochromic indicators, but, as discussed below, are general problems shared among all optical indicators.

2.3. High affinity fluorescent indicators

Tsien and his collaborators synthesized a series of fluorescent Ca^{2+} indicators that have useful properties for biological applications (Grynkiewicz et al., 1985; Minta et al.

1989). In particular, fura-2 and indo-1 have been widely used in many types of cells. Fura-2 and indo-1 have dissociation constant values in the sub-micromolar range ("high affinity" indicators), which makes these indicators useful to measure low $[Ca^{2+}]_i$ near resting levels. During the large Ca^{2+} transients of skeletal muscles, on the other hand, these indicators are likely saturated, particularly in the localized space near the terminal cistern of the sarcoplasmic reticulum, the site of Ca^{2+} release. It has been also reported that fura-2 cannot track fast Ca^{2+} transients of skeletal muscle fibers because of slow kinetics due to its small dissociation rate constant (Baylor and Hollingworth, 1988). Therefore, these high affinity indicators are advantageous for measurements of resting $[Ca^{2+}]_i$, but not suitable for tracking large and fast Ca^{2+} transients of skeletal muscles. However, the high affinity indicators appear to be useful for cell types other than skeletal muscles, such as cardiac and smooth muscle cells as well as non-muscle cells. Fluo-3 has been usefully applied in combination with con-focal imaging to detect localized increase in $[Ca^{2+}]$, e.g., Ca^{2+} sparks (Cheng et al., 1993).

2.4. Low affinity indicators

Chandler and his colleagues first suggested that Ca^{2+} indicators that have a low affinity for Ca^{2+} ("low affinity" indicators) may be useful for measurements of Ca^{2+} transients in skeletal muscle fibers (Maylie et al., 1987b). Southwick and Waggoner (1989) synthesized two purpurate indicators: purpurate-3,3'-diacetic acid (PDAA) and 1,1'-dimethylpurpurate-3,3'diacetic acid (DMPDAA), analogues of murexide and tetramethylmurexide, respectively. These indicators have a low affinity for Ca^{2+} (K_D 0.8-1.0 mM range), and PDAA is highly selective for Ca^{2+} over Mg^{2+} and H^+ (Hirota et al., 1989). When applied to frog cut muscle fibers, both PDAA and DMPDAA are thought to track cytoplasmic Ca^{2+} transients without kinetic delay. The time course of the Ca^{2+} transients was found to be surprisingly fast; time to peak ~5 ms, halfwidth 7-9 ms (16°C). Similar results were obtained in intact frog skeletal muscle fibers injected with PDAA (Konishi and Baylor, 1991). Interestingly, these new indicators were also reported to have a large amplitude for the cytoplasmic Ca^{2+} transients, as large as 20 μM, stimulated by an action potential. Another important advantage of these new purpurate indicators is that the percentage of the indicator that appeared to be bound to intracellular constituents is relatively small (~20% in cut fibers, Hirota et al., 1989; 24-43% in intact fibers, Konishi and Baylor, 1991), in comparison with the large bound fraction (~60-90%) detected with the other indicators. Because an indicator with a smaller bound fraction is likely to yield a more reliable estimate of the amplitude of the cytoplasmic Ca^{2+} transients, the amplitude of Ca^{2+} transients is considered to be more reliable when estimated with PDAA (or DMPDAA) than other indicators.

Recently, many low-affinity fluorescent Ca^{2+} indicators have become available. Furaptra is one of the low-affinity tricarboxylate indicators; although originally designed as a Mg^{2+} indicator (Raju et al., 1990), it has often been used in muscle fibers as a Ca^{2+} indicator with rapid kinetics (Konishi et al., 1991; Claflin et al., 1994; Hollingworth et al., 1996). Although this indicator is commercially available under the name 'mag-fura-2' (Molecular Probes, Inc., Eugene, OR, USA), it has 1.5-2 orders higher affinity for Ca^{2+}

(K_D ~50 μM at 16-37°C) than for Mg^{2+} (K_D = 5.3 mM and 1.5 mM at 16°C and 37°C, respectively)(Raju et al., 1990; Konishi et al., 1991). As expected from these K_D values, furaptra was found to be a useful indicator for measuring the cytoplasmic [Mg^{2+}] in resting muscle fibers (Konishi et al., 1993) and as a Ca^{2+} indicator in stimulated fibers (Konishi et al., 1991). In frog muscle fibers, the brief time course of the cytoplasmic Ca^{2+} transient reported with furaptra is very similar to that reported with PDAA. Thus, furaptra seems to be a useful tool to track the cytoplasmic Ca^{2+} transient without kinetic delay. As in frog fibers, furaptra appears to be useful for tracking the brief time course of Ca^{2+} transients in mammalian skeletal muscle fibers, which generally have diameters about half as large as frog fibers. The cytoplasmic Ca^{2+} transients measured in mouse extensor digitorum longus muscle appear to have a very brief time course, even briefer than those in frog fibers (Hollingworth et al., 1996); after single action potential stimulation, the halfwidth is 4.6 ms at 16°C, and 2.0 ms at 28°C. The results further necessitate the use of low-affinity indicators to faithfully monitor the time course of the cytoplasmic Ca^{2+} transients in skeletal muscle fibers. Thus, our current understanding is that Ca^{2+} transients in frog and mammalian skeletal muscle fibers are large in amplitude (~10-20 μM) and very brief in time course, with a time to peak of ~5 ms and a halfwidth of <10 ms.

3. MEASUREMENTS OF RESTING $[Ca^{2+}]_I$

Two different types of methods have been applied to estimate the resting levels of $[Ca^{2+}]_i$: Ca-ISEs and optical indicators (for the list of estimated resting $[Ca^{2+}]_i$, see Table 1 of Konishi, 1998). Ca^{2+}-selective microelectrodes (Ca-ISEs) are prepared by filling the tip of glass microelectrodes with Ca^{2+} exchange resins. Among the Ca^{2+} exchange resins introduced, the neutral carrier, ETH-1001, has been used most successfully. Tsien and Rink (1980) applied Ca-ISEs with the neutral carrier to frog muscle fibers, and showed that the Ca-ISEs can be used to measure $[Ca^{2+}]_i$. The output of the electrodes should be directly related to the [Ca^{2+}] at the tip; ideally ~30 mV change for a 10-fold change in Ca^{2+} activity. However, at very low Ca^{2+} levels around resting $[Ca^{2+}]_i$ and in the presence of ions which interfere with the electrodes (most importantly K^+, Na^+ and Mg^{2+}), the response of actual electrodes shows much shallower slopes than that expected from Nernstian behavior (for review, see Blinks et al., 1982). Thus, the small errors in the estimation of the cytoplasmic concentration of interfering ions and in membrane potential subtraction lead to a non-negligible difference in the estimation of Ca^{2+} concentration. Another potential source of error of the Ca-ISEs is the leakage of Ca^{2+} around the sites of mechanical impalement, which tends to elevate the local [Ca^{2+}]. Some of the estimates obtained with Ca-ISEs may be erroneously high as a consequence of local membrane damage, as pointed out by Weingart and Hess (1984).

Aequorin is weakly luminescent even in the virtual absence of Ca^{2+} (Ca^{2+}-independent luminescence), but emits large additional luminescence upon Ca^{2+} binding (Allen et al., 1977). The difficulty in the use of aequorin for determination of resting $[Ca^{2+}]_i$ is that the fractional light level obtained from resting muscle is very close to the Ca^{2+}-independent luminescence; even a small error in the in vitro calibration curve greatly influences estimates of $[Ca^{2+}]_i$. Blatter and Blinks (1991) also found that the

fractional luminescence levels of most resting fibers are below the Ca^{2+}-independent luminescence of the calibration curve, giving apparently 'negative' values for $[Ca^{2+}]_i$. A possible reason for the 'negative' estimates for $[Ca^{2+}]_i$ is that myoplasm contains a diffusible macromolecule that interacts with aequorin to reduce Ca^{2+}-independent light emission (Blatter and Blinks, 1991). Thus, to date only upper limit estimates of resting $[Ca^{2+}]_i$ have been obtained from aequorin under experimental conditions in which $[Ca^{2+}]_i$ was raised (and L/L_{max} was increased well above the Ca^{2+}-independent level) either by partial depolarization (Blatter and Blinks, 1991) or application of caffeine (Konishi and Kurihara, 1987).

Fura-2 and indo-1 are the high affinity Ca^{2+} indicators. The high Ca^{2+}-affinity of these indicators is advantageous for monitoring $[Ca^{2+}]_i$ around resting levels. As the vast majority of measurements of $[Ca^{2+}]_i$ are made with fluorescent high affinity dyes, only a few studies have provided useful information on the precise $[Ca^{2+}]_i$ values in muscle fibers. This is primarily because there are difficulties related to calibration of indicator signals in terms of $[Ca^{2+}]_i$. In general, Ca^{2+} indicator molecules are heavily bound to cellular constituents (possibly proteins) within the cytoplasm (Konishi et al., 1988). This binding alters both spectral and binding properties of the indicators; in vitro studies show that the binding to soluble proteins changes both fluorescence and Ca^{2+} binding of fura-2 (Konishi et al., 1988), indo-1 (Baker et al., 1994), fluo-3 (Harkins et al, 1993) and fura-red (Kurebayashi et al, 1993). In frog skeletal muscle fibers, the fluorescence spectra of fura-2 is shifted to longer wavelengths (Konishi and Watanabe, 1995), and as a consequence, a simple comparison of the fluorescence ratio of fura-2 in the cytoplasm and in salt solutions lacking proteins indicates 'negative' $[Ca^{2+}]_i$ (Suda and Kurihara, 1991). Thus, the simple assumption that the indicators behave in the same way in the intracellular environment as in salt solutions is probably not valid, necessitating calibration in the 'intracellular' environment.

Technical difficulties in calibrating optical signals probably contribute to the large discrepancies between recent estimates of $[Ca^{2+}]_i$ in frog muscle at rest (range between <50 nM (Blatter and Blinks, 1991) and 300 nM (Kurebayashi et al., 1993). In an attempt to more accurately estimate resting $[Ca^{2+}]_i$, Konishi and Watanabe (1995) injected fura-2 conjugated to high molecular weight dextran (fura dextran, MW ~10,000) into frog skeletal muscle fibers. To calibrate the indicator's fluorescence in terms of $[Ca^{2+}]_i$, we applied β-escin to permeabilize the cell membrane. After β-escin treatment, small molecules (e.g., Ca^{2+}, ATP etc.) could quickly permeate the cell membrane, whereas 10-kD fura dextran only slowly leaked out from the fiber. It was thus possible to calibrate the indicator fluorescence within the fibers by adjusting the bath solution $[Ca^{2+}]$ to various levels. From the calibration obtained in the β-escin treated fibers, resting $[Ca^{2+}]_i$ in frog skeletal muscle fiber was, on average, 60 nM. Thus it seems reasonable to assume that resting $[Ca^{2+}]_i$ in frog skeletal muscle fibers is within 50-100 nM range.

4. RELATION BETWEEN $[Ca^{2+}]_i$ AND FORCE

The steady-state relation between $[Ca^{2+}]$ and force has been studied primarily with skinned fibers in which the cell membrane is removed to easily establish the known

levels of $[Ca^{2+}]$ in the myofibrillar space (Natori, 1954; Endo and Iino, 1980). In spite of the usefulness of skinned fibers, it is desirable to obtain a quantitative relation between $[Ca^{2+}]_i$ and force in intact muscle fibers, because removal of the cell membrane should produce a large alteration in the environment surrounding the myofibrils and may well influence the $[Ca^{2+}]$-force relation.

In intact muscle fibers stimulated by either a single action potential or a train of action potentials, force generation follows a rapid rise and fall of $[Ca^{2+}]_i$ with a significant delay, forming a non-equilibrium relation between $[Ca^{2+}]_i$ and force. However, under experimental conditions that significantly slow the change in $[Ca^{2+}]_i$ to reach equilibrium with force generation, simultaneous measurements of $[Ca^{2+}]_i$ and force should provide information on the steady-state relation between $[Ca^{2+}]_i$ and force. Konishi and Watanabe (1998) measured $[Ca^{2+}]_i$ with fura dextran (see above) in frog skeletal muscle fibers, and plotted $[Ca^{2+}]_i$ versus force during high K^+ contractures and during the slow relaxation phase after tetani in the presence of an inhibitor of the SR Ca^{2+} pump. The $[Ca^{2+}]_i$-force data obtained from the two types of measurements agreed fairly well, consistent with a Hill coefficient of 3.2-3.9 and a Ca_{50} of 1.5-1.7 μM (sarcomere length 2.6-2.8 μm, 16-18°C). We concluded that the steady state force was a 3rd to 4th power function of $[Ca^{2+}]_i$, and half maximal force was achieved in the low micromolar range in intact frog skeletal muscle fibers. However, there are still significant contradictions and uncertainties concerning the steady state relation between $[Ca^{2+}]_i$ and force in intact skeletal muscles. Clearly, further improvements for measurements of both $[Ca^{2+}]_i$ and force are necessary to resolve this important issue.

5. INHOMOGENEOUS ACTIVATION WITHIN SARCOMERES

In the previous sections, $[Ca^{2+}]_i$ refer to the spatially averaged $[Ca^{2+}]$ in the cytoplasm. In the steady state, $[Ca^{2+}]$ levels in the sarcomeres are thought to be uniform. However, when Ca^{2+} is released from the terminal cistern of the SR, the concentration gradient of cytoplasmic free Ca^{2+} should develop. When the SR Ca^{2+} release is turned off, cytoplasmic Ca^{2+} diffusion causes the gradient to dissipate. During the Ca^{2+} transient, therefore, the local level of $[Ca^{2+}]$ within the sarcomeres is higher or lower than $[Ca^{2+}]_i$, in accordance with the distance from the Ca^{2+} release channels of the SR and the time after initiation of the release. Hollingworth et al. (2000) experimentally confirmed the sarcomeric Ca^{2+} gradient during twitch activation of frog skeletal muscle fibers imaged with two-photon microscopy. They found that the time course of fluo-3 fluorescence changes was very different at the z- and m-lines, with a significantly delay (about 1 ms) between the rise of fluorescence at the z-line (near the terminal cistern) and the m-line (the middle of sarcomeres). These localized changes in $[Ca^{2+}]_i$ which would lead to non-uniform activation of myofilaments are complex functions of intrasarcomeric geometry and time, and their significance requires clarification by future studies.

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DISCUSSION

Gonzalez-Serratos: 1) We have reported that, during activation of isolated frog muscle fibers, Ca^{2+} is released as spots that appear and disappear continually during all the period of titanic activation. 2) You showed that, with 30 mM [K^+], the force is nearly maximum. However, it has been shown by several researchers including me, that 30 mM [K^+] produce only around 1 /3 of the maximum force. Do you think that the calcium probe you are using may change contractility?

Konishi: I have seen your abstract in the Biophysical Society, although I did not know if it was published as a full paper. Probably I should have referred to your work. However, what I liked to emphasize in this talk was non-uniformity within a sarcomere. Non-uniformity within a fiber that you pointed out is another important factor to modify fiber activity. We observed some fiber-to-fiber variation of the [K^+]-force relation, but you may be right that we have in general slightly more force generation at 30mM [K^+] than that reported in the literature. We do not think that the relation was modified in the presence of fura-dextran, because we obtained very similar results in the fibers without indicator infection or the fibers injected with aequorin. I don't know what makes the difference, and I can only say that this is what we observed under our experimental conditions. We prepared high K^+ solutions of constant [K^+]x[Cl^-] product by equimolar substitution of NaCl with K-methanesulfonate.

Maughan: How large was the dextran probe?

Konishi: 10 K mol wt.

Maughan: A comment: you mentioned calcium spatial heterogenity reflected by Ca^{2+} sparks. Another source of calcium spatial heterogenity is the gradient set up by the presence of highly negatively charged myofilaments, where the Ca^{2+} concentration is much greater in the vicinity of the thin filaments and troponin C, and when the calcium probe Fula-2, a highly charged anion is excluded to some extent from the vicinity of the troponin. Have you done calculations to assume the degree of what your in vivo force P-Ca curves are modified to reflect external conditions at the troponins?

Konishi: Simple calculation of the Donnan equilibrium predicts that the intramyofibrillar Ca^{2+} concentration would be somewhat higher than the extramyofibrillar concentration. However, the myofilament charges might not fully influence the Ca^{2+} indicator molecules between the myofilament separated by about 40nm, which is a distance much greater than the Debye length of about 1nm. This is a very difficult situation to model.

Hexley: What is the dissociation of troponin for Ca^{2+}? Does binding constant of troponin (in these works for calcium agree with free Ca^{2+} level needed for activation?

Konishi: A dissociation constant of troponin is generally assumed to be about 2 μM, which is consistent with the findings that Ca^{2+} transients are large in amplitude. However, attachment of myosin head significantly lowers the troponin dissociation constant, so the situation is not simple and not totally clear.