A probable role of dihydropyridine receptors in repression of Ca\textsuperscript{2+} sparks demonstrated in cultured mammalian muscle

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In striated muscle, contraction is triggered by Ca\textsuperscript{2+} release from the sarcoplasmic reticulum (SR) through ryanodine receptor (RyR) channels. The state of these channels is determined by the action potential by means of dihydropyridine receptors (DHPRs), voltage sensors located in the T tubular membrane. Most current studies of this regulation focus on the activation or opening of the release channels, which is known to require an initial action, most probably mechanical, by the voltage sensors. Ca\textsuperscript{2+} release initiated thus may then be reinforced by Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release (CICR), a secondary activation mediated by Ca\textsuperscript{2+} itself (14, 21). In addition to these positive actions by voltage sensors and Ca\textsuperscript{2+} ions, there is evidence of an inhibitory mechanism that is “dynamic,” brought to bear only after Ca\textsuperscript{2+} channel opening and Ca\textsuperscript{2+} release (2, 36, 43, 46). This inhibition is thought to be crucial for maintaining stability and speed of control in a system whose activation may be highly self-sustaining as a consequence of CICR, and appears to be also mediated by Ca\textsuperscript{2+}.

Although much attention has been paid to this dynamic inhibition/inactivation mechanism, some evidence has emerged of a role of DHPRs in inhibition of release channels.

Initial indications of a basal inhibition by DHPRs originated from comparisons of excitation-contraction (EC) coupling between amphibian and mammalian muscle. The comparisons led Shirokova et al. (51) and Zhou et al. (67) to conclude, in agreement with earlier studies (15, 16), that CICR has a limited or null role in mammalian muscle. An important contribution of CICR, however, was upheld for frog muscle.

The apparent differences in the contributions of CICR between amphibians and mammals ought to be explained by the different molecular makeup and supramolecular assembly of their EC coupling devices. Whereas frog fast-twitch muscles contain the \(\alpha\) - and \(\beta\)-isofoms, in approximately equal densities (42, 56), most muscles of adult mammals exclusively express the \(\alpha\)-homolog, RyR1 (19, 34, 58), whereas some additionally express the \(\beta\)-homolog, RyR3, in minor amounts.

According to Felder and Franzini-Armstrong (17), \(\alpha\)RyRs form separate arrays of channels, placed parajunctionally, on the sides of the triadic junction. Junctional clusters are instead constituted exclusively by the \(\alpha\) (or, in mammals, the RyR1) isoform. Because only the latter are associated with tetrads of DHPRs in the T tubule membrane (3), the activation mechanisms of these two sets of channels must be distinct (67). The parajunctional channels, which lack directly interacting DHPRs, can be under voltage control only indirectly, presumably by CICR. Conversely, the location of junctional channels should allow them to be directly controlled by voltage sensors. In agreement with these functional expectations, studies in IBS RyR-deficient myotubes show that RyR1 can be controlled by membrane voltage, whereas RyR3, if expressed alone, can only be activated via CICR (18).

The notion of a basal inhibition originated in good measure from the very different incidence of Ca\textsuperscript{2+} sparks in muscles of frogs and mammals. Ca\textsuperscript{2+} sparks are discrete local elevations of Ca\textsuperscript{2+} concentration (\([\text{Ca}^{2+}]_i\)), which reflect opening of a group of RyR channels spontaneously or in response to voltage

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or other stimuli (6, 61). The activation of multiple channels in Ca\(^{2+}\) sparks is believed to involve Ca\(^{2+}\) as mediator. This consensus derives largely from evidence collected in frog muscle, including that sparks are promoted by increases in resting cytosolic [Ca\(^{2+}\)] (f[Ca\(^{2+}\)]\(_{cyt}\)) (28), by increases in SR Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_{SR}\)), and by caffeine (24), which increases RyR sensitivity to activation by Ca\(^{2+}\) (15, 35).

In contrast with amphibians, sparks are rarely observed (10) or not found at all (66) in intact adult skeletal muscle cells of mammals, and are not elicited in adult cells by voltage-clamp depolarization (11, 52). Spark frequency, however, becomes much greater under other conditions, including membrane permeabilization by saponin or its removal by “peeling” (27, 66) and interference with mitochondrial function (26). Therefore, sparks are possible in the mammal, but appear to be suppressed. Comparison of spark frequency in IB5 dyspedic cells expressing alternatively either isoform demonstrated that the suppression affects largely isoform 1 (62).

Suppression of a related sort was revealed by Murayama and Ogawa (37, 38) affecting bovine RyR1 and amphibian α isoforms. Because β was not affected, the stabilization may explain the greater contribution of CICR in the amphibian. The authors provided evidence pointing to FKBP12 as a possible effector of this inhibition in mammalian muscle.

The inhibition has an intriguing functional feature. Using primary mammalian myotubes, Shirokova et al. (53) showed dual spatially segregated manifestations of Ca\(^{2+}\) release. Whereas some regions of the myotube featured strict control of Ca\(^{2+}\) release by membrane voltage (of the “skeletal” type, i.e., not dependent on Ca\(^{2+}\) entry), other regions did not respond to voltage, but produced Ca\(^{2+}\) sparks essentially unrelated to the depolarizing pulses. These two forms of Ca\(^{2+}\) release appear to be mutually exclusive (regions that respond to voltage do not produce sparks), which suggests that something in the devices underpinning voltage control, perhaps the DHPR itself, suppresses spark generation. Consistent with these ideas, Lee et al. (33) provided evidence of an inhibitory allosteric effect in IB5 myotubes. A putative molecular locus underpinning the suppression is indicated by the existence of a malignant hyperthermia-inducing mutation in the III-IV loop of the DHPR in humans (65).

Motivated by these varied observations, the present study set out to test whether T tubules play a role in the suppression of sparks. We also explored, using myotubes lacking DHPRs, whether the voltage sensors themselves are needed for the inhibition. Because the production of sparks in mdg muscle required the use of special nonphysiological conditions, we used the shifted excitation and emission ratio (SEER) imaging technique (31) to compare [Ca\(^{2+}\)]\(_{cyt}\) and intrastore [Ca\(^{2+}\)] in both types of cells under those conditions.

METHODS

Cultures of skeletal muscle myotubes. Primary cultures were prepared from the skeletal muscle of neonatal mice (postnatal day 0) of the stock CACNA1SxNIHS-BCfBR (39, 47). Myotubes from wild type (mdg/+) or heterozygotes (mdg+/−) were undistinguishable. Dysgenic or mdg myotubes (mdg+/−) lack DHPRs in the T tubules (29) and lack EC coupling (59).

Muscle tissue was dissected from the limbs and digested with 2 mg/ml collagenase I (Worthington Biochemical, Lakewood, NJ) in 0 Ca\(^{2+}\)-0 Mg\(^{2+}\) PBS (GIBCO, Carlsbad, CA) at 37°C for 30 min. Myoblasts were collected by passing the suspension through a 40-μm-thick nylon filter (Falcon, Bedford, MA), seeded in glass-bottomed microwell dishes (MatTek, Ashland, MA), and maintained in the growth medium (DMEM, 20% FBS, GIBCO) for 2 days before they differentiated into myotubes in the differentiation medium (DMEM, 2.5% horse serum). Images were obtained from cultures that underwent differentiation for 2–4 days. Experiments were performed in compliance with National Institutes of Health guidelines and were approved by the Institutional Animal Care and Use Committee of Rush University.

Solutions. The Krebs solution was composed of (in mM) 136 NaCl, 5 KCl, 2.5 CaCl\(_2\), 1 MgCl\(_2\), 10 HEPES, and 10 glucose, pH 7.0. High-Ca\(^{2+}\) Krebs (K/Ca) was composed of (in mM) 118 NaCl, 5 KCl, 26 CaCl\(_2\), 1 MgCl\(_2\), 10 HEPES, and 10 glucose, pH 7.0, caffeine (K/Ca+C), with 50 μM nifedipine (K/Ca+N), or both (K/Ca+N+C), were added for different purposes. All solutions contained 1 μM tetrodotoxin to prevent spontaneous activation.

For dye loading, cell cultures were immersed in Krebs solution with 5 μM of either indo-1 AM or mag-indo-1 AM. For membrane permeabilization, cultures were immersed in an internal solution (containing in mM) 130 K-glutamate, 10 Trizma maleate, 5 Na\(_2\)ATP, 10 Tris-PC, 1 EGTA, 5 glucose, 8% dextran, 0.1 CaCl\(_2\), 7.23 MgCl\(_2\) (free Ca\(^{2+}\) = 50 nM, free Mg\(^{2+}\) = 1 mM), and 0.05 rhod-2 plus 0.002% saponin.

The solutions were prepared and kept at 18–20°C. Chemicals for these solutions were purchased from Sigma-Aldrich (St. Louis, MO).

Optical measurements and data analysis. For detection of spontaneous Ca\(^{2+}\) sparks, cultures were incubated with 5 μM fluo-4 AM (Molecular Probes, Eugene, OR) in Krebs solution for 50 min at 20°C, then washed and maintained in fresh Krebs solution for an additional 40 min. For double-staining experiments, the cultures were exposed to 10 μM 4-[2-(6-[dioctylamino]-2-naphthalenyl)-2-naphthalenyl]-1[3-sulfopropyl]-pyridinium (di-8-ANEPPS) (Molecular Probes) for <15 min to minimize its entry into organelles. The dye was then washed away with Krebs solution.

Cells were imaged with a confocal scanner equipped with a ×40 water-immersion objective (model MRC-1000, Zeiss; numerical aperture 1.2). Dual images were obtained with excitation light of 488 nm and simultaneous recording at × = 530 nm (±25-nm bandwidth) for detection of cytosolic Ca\(^{2+}\) events, and × > 588 nm for detection of structures stained by di-8-ANEPPS. Ca\(^{2+}\) events were detected automatically in x or xy images and normalized as described by Brum et al. (4). The detection program carries out automatic determination of spark parameters: amplitude (peak F/F\(_{0}\)), full width above half magnitude (FWHM), full duration above half magnitude (FDHM), and rise time (from 10% to full peak at the spatial center of the spark) (66). The location of sparks in myotubes relative to the surface membrane was represented by a number that quantified it relative to the cell radius, with 0 corresponding to the membrane and 1 to the cell center. The fractional radius of the myotube invaded by T tubules (f\(_{T}\)) was determined by comparison of T-tubule-free and occupied areas (dashed line in Fig. 1B) with midcell xy images of doubly stained myotubes.

SEER imaging of myotubes. Two applications were implemented: determination of [Ca\(^{2+}\)]\(_{cyt}\) in intact cells loaded with indo-1 and imaging of [Ca\(^{2+}\)]\(_{ins}\) in membrane-permeabilized cells with mag-indo-1 inside organelles.

SEER (31) required the simultaneous acquisition of two confocal fluorescence images (named F\(_{1}\) and F\(_{2}\) produced by alternating line by line (“line interleaving”) two excitation lights (351 and 364 nm) with Krebs solution.

Two applications were implemented: determination of [Ca\(^{2+}\)]\(_{cyt}\) in intact cells loaded with indo-1 and imaging of [Ca\(^{2+}\)]\(_{ins}\) in membrane-permeabilized cells with mag-indo-1 inside organelles.
ages $F_{11}(x,y)$ and $F_{22}(x,y)$ of cells loaded with indo-1 AM are provided in Fig. 6, A and B. Images of a permeabilized cell loaded with mag-indo-1 AM are shown in Figs. 9, A and B, and 10B. $F_{33}$ is shown in Figs. 9 and 10. All other images are of the membrane permeabilized, so that the dye remnant inside the cell was not stained (in other areas color was set to gray). The well-stained areas were those with dye concentration comprised between the mean and the mean plus three times the standard deviation of the pixel-by-pixel distribution of dye concentration within the image. Dye concentration was calculated from a linear combination of $F_{11}$ and $F_{22}$ (31).

**Imaging of $[\text{Ca}^{2+}]_{\text{jcyt}}$.** The technique was essentially that of Launikonis et al. (31). Cells loaded with mag-indo-1 had their plasma membrane permeabilized, so that the dye remnant inside the cell was all trapped in organelles, largely the SR. $[\text{Ca}^{2+}]_{\text{jcyt}}$ is derived from the ratio through pixel-by-pixel application of the equation

$$[\text{Ca}^{2+}] = K_D \beta \frac{R - R_{\min}}{R_{\max} - R}$$

(1)

where $R_{\min}$ and $R_{\max}$ are minimum and maximum ratio values, $K_D$ is the dissociation constant of the dye, and $\beta$ is the ratio of $F_{22}$ at $[\text{Ca}^{2+}] = 0$ to $F_{22}$ at saturating $[\text{Ca}^{2+}]$. Parameter values are given by Launikonis et al. (31). *Equation 1* and the equation published by Grynkiewicz et al. (25) differ only in the definition of $\beta$.

**Measurement of $[\text{Ca}^{2+}]_{\text{jcyt}}$.** For the measurement of $[\text{Ca}^{2+}]_{\text{jcyt}}$ with indo-1, the culture dishes were loaded and then imaged intact. As with any procedure on intact cells loaded with a dye in AM form, there will be some interference by the dye that accumulates inside organelles. A correction for this error, developed in the Appendix, leads to an equation isomorphic with 1

$$[\text{Ca}^{2+}] = K_{\text{eff}} \beta \frac{R - R_0}{R_{\max} - R}$$

(2)

where $K_{\text{eff}}$ and $R_0$ are effective values of $K_D$ and $R_{\min}$. Their expressions in terms of the other parameters are given in the Appendix.

Calibration experiments to determine the parameters of Eq. 2 were carried out in cultures loaded with indo-1, so that the organelles would cause similar interference as in the intact cells. After incubation with indo-1 AM, the loaded cells were membrane permeabilized and SEER imaged in solutions with known buffered $[\text{Ca}^{2+}]$ and 50 $\mu$M indo-1. $K_{\text{eff}}$ $\beta$ was determined by fitting Eq. 2 to the data points (R, $[\text{Ca}^{2+}]$), where $R$ was the ratio averaged in those areas that satisfy a criterion for good staining described above. The parameter values were $R_0 = 0.51$, $R_{\max} = 3.94$, $K_{\text{eff}} = 1.620$ nM, and $\beta = 4.11$, implying that $K_{\text{eff}}$ was 394 nM.

**Statistics.** Line graphs, such as Fig. 6F, plot averages of SEER ratio values over all well-stained pixels of a cell or cells within an image. The standard errors of such means are uniformly smaller than the symbol size. When a bar is plotted, the symbols represent means over averages of pieces of a cell or image. In those cases, the bar covers means $\pm 2$ SE. Such piecewise approach was necessary to exclude well-stained debris, the inclusion of which would have biased the average. Significance of differences indicated in vertical bar graphs is determined by two-tailed $t$-tests.

**Immunofluorescence staining.** Cultures were fixed for 20 min at $-20^\circ$C with methanol-acetone (1:1) precooled at $-20^\circ$C. Fixed cells were incubated with blocking solution overnight at 4°C or 1 h at room temperature. The blocking solution was PBS (GIBCO) containing 10% normal goat serum, 1% BSA, and 0.1% Triton X-100 (Sigma-Aldrich). The cells were incubated with the primary antibodies, anti-RyR1 (1:1,000) or anti-RyR3 (1:2,000), diluted in the blocking solution for 1 h at room temperature or overnight at 4°C, then incubated with secondary antibodies, Cy2-conjugated goat anti-rabbit (Jackson ImmunoResearch Laboratories, West Grove, PA), in the blocking solution for 45 min at room temperature. Control experiments were done by replacing primary antibody with 1:1,000 normal rabbit serum (Sigma-Aldrich) in blocking solution. The primary antibodies, a gift from Dr. V. Sorrentino (University of Siena, Italy), have been shown not to cross-react with each other (22).

**RESULTS**

**A pattern of location of Ca$^{2+}$ sparks in developing muscle.** A first set of experiments used developing myotubes, in which T tubules were incomplete and largely occupied the peripheral region, to explore whether there was a topographical correspondence between T tubule structures and the occurrence of spontaneous sparks.

In myotubes produced in primary cultures from normal newborn mice, Ca$^{2+}$ sparks were observed consistently at a low frequency. A total of 79 sparks were detected and characterized in $xy$ images from 17 intact cells immersed in Krebs solution. Sparks occurred more frequently in myotubes of larger diameter, such as the one shown in Fig. 1. The culture was dually stained with fluo-4 AM and the membrane-soluble dye di-8-ANEPPS, and images were acquired simultaneously at two emission bands. In Fig. 1A, the fluorescence of fluo-4 in one image is normalized to the average of six other images
from the same region of the specimen. Figure 1B is the averaged fluorescence of di-8-ANEPPS at longer wavelengths in the same region. It allows visualization of T tubules.

Like the one shown in Fig. 1, most Ca^{2+} sparks occurred away from the peripheral region. As shown in Fig. 1B, regions where sparks were frequent did not have structures stained with the membrane marker. A numerical location of the events recorded in these images was defined as the ratio (distance from center of spark to cell edge)/cell radius. Figure 2 shows the histogram of locations of all identified sparks.

The average extent of development of T tubules was measured in images of these and other cells stained with di-8-ANEPPS. The extent of occupancy by T tubules, again as a fraction of cell radius, represented by $f_t$, was calculated as the ratio of T tubule occupied to total area in images such as that in Fig. 1B. The average of this ratio in 9 cells, $0.22 \pm 0.06$, is indicated by a horizontal bar in Fig. 2. Clearly, sparks occurred at a very low frequency in the region occupied by T tubules. When the extent of occupancy by T tubules was calculated as the square root of the ratio of areas, as would befit cells of circular perimeter, the radial extent of occupancy by T tubules was 0.12. Regardless of the approximation used, there was a close correspondence between the radial extent free of sparks and that occupied by T tubules, reflecting a pattern of avoidance of T tubules by sparks.

**Topographic pattern of spark generation is lost in dysgenic myotubes.** If, as shown, the presence of T tubules suppresses the generation of Ca^{2+} sparks, a first candidate as molecular inhibitor ought to be the DHPRs, which interact mechanically with RyR1 channels in the T-SR junction (40). Skeletal muscle cells from mice homozygous for the mdg mutation (47), which lack DHPRs in the T tubules (29), were used to test this hypothesis. Primary cultures of skeletal muscle taken from neonatal mdg^{+/+} pups produced myotubes that were morphologically similar to those of the wild type, but appeared to have a somewhat greater diameter, as described by Powell et al. (48). Figure 3A shows the image of an mdg cell labeled with di-8-ANEPPS. After 3–4 days in differentiation medium, dysgenic myotubes, like their wild-type counterparts, had a partially developed T tubule structure, occupying the peripheral region to an extent similar to that in normal myotubes ($f_t = 0.234 \pm 0.03$, $n = 8$).

No spontaneous Ca^{2+} sparks were detected in dysgenic myotubes in Krebs solution. Caffeine (10 mM) induced a global release, which demonstrated integrity of the Ca^{2+} source (data not shown). Several probable causes for the absence of spontaneous sparks were considered. One could be a low Ca^{2+} load in the SR, which, according to evidence from cardiac and skeletal muscle, would reduce event frequency (60, 67). It could be also a direct consequence of the absence of plasmalemmal L-type Ca^{2+} channels, which have a role in the initiation of Ca^{2+} sparks in embryonic mouse muscle (8), or it could be a consequence of lower cytosolic Ca^{2+}. All these possibilities were tested by direct measurements, described below.

To induce spark production, the cultures were exposed to K/Ca^{2+}C, a modified Krebs with 26 mM Ca^{2+} and a low dose of caffeine (1 mM). As shown in Fig. 3B, 315 sparks were detected and characterized in $xy$ images from 24 dysgenic myotubes. The most remarkable difference with the normal cells was that sparks could occur just inside the plasmalemma. As shown in Fig. 3A, T tubules could be visualized well in cells stained with di-8-ANEPPS. The spatial location of the events, represented by the histogram in Fig. 4, bore little or no relation with the location of T tubules (represented by the horizontal bar). Therefore, in dysgenic cells, the suppression effect on the releasing channel opening was lost under the conditions required to observe sparks. This absence could reflect a fundamental molecular influence or just a trivial artifact.

Ca^{2+} overload erases the pattern of spark suppression in normal cells. The lack of a topographic pattern of spark generation in mdg myotubes might be simply a consequence of Ca^{2+} overload, which could occur in a dysgenic myotube in the high-Ca^{2+} overload (of cytosol or intracellular stores) in the high extracellular [Ca^{2+}] ([Ca^{2+}]_o) needed to induce spontaneous sparks. A first control for this possibility was a study of the spatial distribution of sparks in normal myotubes. The cells were exposed to K/Ca^{2+}C, the high-Ca^{2+} Krebs. One hundred and forty-two sparks were detected in $xy$ images from twenty-one cells. As shown by the histogram in Fig. 5A, the pattern of locations was lost, with sparks appearing equally frequently in all areas of the cells. According to this result, the loss of the spatial pattern of spark origination in mdg cells could indeed be a consequence of Ca^{2+} overload. A more specific control was introduced, as described next.

**Spatial pattern is maintained in high [Ca^{2+}] if L-type channels are blocked.** It is reasonable to expect that a normal myotube in a high-Ca^{2+} extracellular solution will acquire a higher Ca^{2+} load (in cytosol and stores) than a dysgenic myotube in the same solution, as L-type channels present in the normal cell should provide additional paths for entry of Ca^{2+}. An alternative control condition was designed with the aim of better reproduction of the membrane permeability of dysgenic myotubes in wild-type cells. Sparks were studied in normal myotubes in the high-Ca^{2+} solution K/Na+NC, which included nifedipine, a specific L-type channel blocker (41) with no direct effect on release channels (64). As shown later, in

![Fig. 2. Spatial location of spontaneous sparks of myotubes in Krebs solution. Distance of the spark source from the cell surface along the scanning line is normalized to cell radius, with 0 corresponding to the membrane and 1 to the cell center. The bars at top represent the averaged value in 9 cells ($0.22 \pm 0.06$) of $f_t$, the fractional development of T tubules. The histogram shows the distribution of 79 sparks detected in $xy$ images from 19 cells.](http://ajpcell.physiology.org/)

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experiments using SEER to monitor $[\text{Ca}^{2+}]$, these conditions lead to similar concentrations in the compartments of interest in mdg and normal myotubes.

The histogram of locations of 361 sparks from 26 normal myotubes in K/Ca+NC is plotted in Fig. 5B. It shows that the pattern of avoidance of T tubules by sparks, which was lost in mdg cells, persisted in normal cells under these conditions. The averaged morphological parameters of 165 events detected in mdg pattern of avoidance of T tubules by sparks, which was lost in $\text{Ca}^{2+}$/H11001 myotubes in K/Ca NC is plotted in Fig. 5.

Measurements of cytosolic and SR-luminal $[\text{Ca}^{2+}]$. Measurements were carried out using SEER (31) to compare the state of the $[\text{Ca}^{2+}]$ of interest under the special conditions used to elicit $\text{Ca}^{2+}$ sparks. The study followed the evolution of $[\text{Ca}^{2+}]_{\text{cyto}}$ and $[\text{Ca}^{2+}]_{\text{SR}}$ as the extracellular solution was changed from Krebs to the high-$[\text{Ca}^{2+}]$ K/Ca with or without nifedipine and/or caffeine.

Evolution of $[\text{Ca}^{2+}]_{\text{cyto}}$. Figure 6, A–C, shows images F11, F22, and R ($=F_{11}/F_{22}$) of wild-type myotubes in Krebs solution. Figure 6, D and E, show ratio images in subsequent stages of work with the same culture dish. The average ratio in Fig. 6C corresponds to a $[\text{Ca}^{2+}]_{\text{cyto}}$ of 152 nM. When the solution was changed to K/Ca with 50 µM nifedipine and 1 mM caffeine, the ratio did not change (Fig. 6D). After a later change to K/Ca without nifedipine, the ratio increased substantially, eventually reaching a value corresponding to $[\text{Ca}^{2+}]_{\text{cyto}}$ of 1.3 µM (Fig. 6E). As shown, $[\text{Ca}^{2+}]_{\text{cyto}}$ could then be very different in different cells. Figure 6F plots the time course of image-averaged ratio (the right-side axis is labeled with the corresponding $[\text{Ca}^{2+}]$ values, calculated using Eq. 2 in Methods).

Corresponding ratio images for cultures of dysgenic cells are illustrated in Fig. 7. $[\text{Ca}^{2+}]_{\text{cyto}}$ in Krebs varied near 170 nM. Upon changing the solution to 26 mM (no caffeine), $[\text{Ca}^{2+}]_{\text{cyto}}$ remained close to initial values. The addition of 1 mM caffeine appeared to cause a small increase in some cells. Figure 7D plots the time course of image-averaged ratio and $[\text{Ca}^{2+}]_{\text{cyto}}$.

Figure 8 summarizes results with various solutions. Wild-type and dysgenic cells immersed in Krebs had similar $[\text{Ca}^{2+}]_{\text{cyto}}$, 152 and 171 nM, respectively. In Krebs with 26 mM $\text{Ca}^{2+}$, $[\text{Ca}^{2+}]_{\text{cyto}}$ increased significantly in the wild type, to 464 nM, but in dysgenic cells it did not (175 nM). The change in the wild type was essentially suppressed when nifedipine was added to the high-$\text{Ca}^{2+}$ Krebs (166 nM).

As reported there, sparks in high-$\text{Ca}^{2+}$ Krebs plus caffeine occurred at random locations in mdg cells, whereas in normal myotubes (in the same solution plus nifedipine) events failed to occur at peripheral locations. As shown in Fig. 8, these conditions led to a somewhat lower $[\text{Ca}^{2+}]_{\text{cyto}}$ in mdg (118 nM vs. 192 nM in the wild type). This difference makes it impossible
that the loss of the pattern of spark generation is due to an increased stimulation by cytosolic Ca\(^{2+}\) in the dysgenic cells.

**Comparisons of SR luminal \([Ca^{2+}]_\text{SR}\).** Figure 9 illustrates the technique. Figure 9, A and B, are images of a cell in a culture dish loaded with mag-indo-1 AM and then membrane-permeabilized. Figure 9C is the ratio \(F_{11}/F_{22}\), restricted as described in METHODS to areas of the cell where the dye concentration was above the cellular mean. This restriction essentially removes nuclei, as well as debris, from the ratio image. The image in Fig. 9D is of fluorescence \(F_{33}\) of the dye rhod-2, present in the solution to identify permeabilized myotubes.

In principle, the permeabilization step reduces one’s ability to follow changes in \([Ca^{2+}]_\text{SR}\) upon changing extracellular solutions because the SR contents may vary after the plasma membrane is permeabilized. The approach illustrated in Fig. 10 was designed to evaluate the evolution of \([Ca^{2+}]_\text{SR}\) after permeabilization. Figure 10A shows fluorescence of rhod-2, in an \(mdg\) culture dish preloaded with mag-indo-1 AM and immersed in the permeabilizing solution (which contains 50 nM free Ca\(^{2+}\)). Figure 10B is fluorescence \(F_{22}\) of mag-indo-1, which is present in cytosol and organelles. A very bright cell in Fig. 10B corresponds to a dark region of Fig. 10A (dashed contour), marking a myotube that has not been permeabilized. The myotube that is highly fluorescent in Fig. 10A has much lower \(F_{22}\) intensity in Fig. 10B. The cellwide concentration of mag-indo drops \(\sim 10\) times after permeabilization, consistent with the presence of much more dye in the cytosol than in organelles. Figure 10C shows the ratio with a high value in the permeabilized cell, consistent with the high \([Ca^{2+}]_\text{cyto}\) in the SR, and a low value in the intact cell, where the dye signal is largely determined by \([Ca^{2+}]_\text{cyto}\).

The heterogeneous staining pattern of Fig. 10, A–C, is characteristic of initial stages of permeabilization. Although it is not possible to know the exact time, most cells become permeabilized in 2–3 min. The evolution of \([Ca^{2+}]_\text{SR}\) can then be followed for tens of minutes after permeabilization, as illustrated in Fig. 10, D–F. Figure 10D is from a wild-type culture in Krebs, and Fig. 10E is from a different wild-type dish, exposed to K/Ca for 10 min before permeabilization. The evolution of \([Ca^{2+}]_\text{SR}\) after permeabilization, plotted in Fig. 10F, is slow. Hence, there is time after permeabilization to obtain several images and derive an average \(R\) that will represent approximately the prevailing level of \([Ca^{2+}]_\text{SR}\) in the conditions imposed before permeabilization. The plot in red is representative of the substantial increase in \([Ca^{2+}]_\text{SR}\) observed in normal myotubes in high Ca\(^{2+}\) Krebs.

Figure 11 summarizes the measurements of \([Ca^{2+}]_\text{SR}\). The ratio measured in wild-type cells immersed in Krebs corresponds to a \([Ca^{2+}]_\text{SR}\) of 82 \(\mu\)M. Immersion in K\(^{+}\)/Ca\(^{2+}\) with nifedipine increased \([Ca^{2+}]_\text{SR}\) significantly, to 144 \(\mu\)M. As expected, immersion in high \([Ca^{2+}]_o\) without the dihydropyridine caused an even greater increase in \([Ca^{2+}]_\text{SR}\), to 284 \(\mu\)M. The \([Ca^{2+}]_\text{SR}\) level of \(mdg\) cells in Krebs, 206 \(\mu\)M, was significantly greater than that of normal cells in the same condition. Immersion of \(mdg\) cultures in K\(^{+}\)/Ca\(^{2+}\) had an unexpected result, \([Ca^{2+}]_\text{SR}\) decreased. Serendipitously, this decrease made the average value in \(mdg\) cells identical to that of wild-type cells in K/Ca+N (K/Ca and K/Ca+N were the

![Graph](#)

**Table 1. Morphological parameters of sparks detected automatically in xy images**

<table>
<thead>
<tr>
<th></th>
<th>(n)</th>
<th>Amplitude</th>
<th>FWHM, (\mu)M</th>
<th>FDHM, ms</th>
<th>Rise Time, ms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>165</td>
<td>0.53±0.03</td>
<td>4.62±0.14</td>
<td>41.5±0.76</td>
<td>29.2±0.66</td>
</tr>
<tr>
<td>(mdg)</td>
<td>1,276</td>
<td>0.41±0.01</td>
<td>4.87±0.05</td>
<td>44.2±0.19</td>
<td>30.2±0.19</td>
</tr>
</tbody>
</table>

Values are means ± SE; \(n\), no. of events detected automatically. \(mdg\), dysgenic mouse myotubes. Parameters, including full width and full duration at half magnitude (FWHM and FDHM), are defined in METHODS. Sparks of \(mdg\) myotubes were recorded in Krebs solution with 26 mM Ca\(^{2+}\) and 1 mM caffeine. Sparks of wild-type myotubes were recorded in Krebs solution with 26 mM Ca\(^{2+}\), 1 mM caffeine, and 50 \(\mu\)M nifedipine.
conditions used for comparing location of sparks in mdg and wild-type cells).

**Location of ryanodine receptor isoforms in wild-type and mdg myotubes** The occurrence of fewer sparks in the periphery of normal myotubes could be due to a paucity of release channels in this area. Although the adult mammalian skeletal muscle predominantly expresses RyR1, developing cells express RyR3 at a high level (54). Ca\(^{2+}\)/H\(\text{11001}\) sparks could originate from either isoform (9, 53, 62), although myotubes expressing RyR3 had a much greater frequency of spontaneous events than those expressing RyR1 (63). To identify the expression of these isoforms, we used antibodies against RyR1 or RyR3. Figure 12, top, shows fluorescence images of myotubes from normal or mdg cultures immunolabeled with anti-RyR1 or anti-RyR3 antibodies. Confocal sections ran near the myotube axis, as evidenced by the presence of nuclei. In normal myotubes, the density of sites reactive to anti-RyR1 antibodies was greater near the surface in some cases (Fig. 12A) and randomly distributed in the cytosol in others (Fig. 12B). The RyR3-specific reactivity was likewise present nearly everywhere, although in some images it was denser near nuclei (Fig. 12C). The distribution pattern of RyR1 and RyR3 in mdg (Fig. 12, D–F) was not visibly different from that of the wild-type cells, including cases, not shown, in which RyR3 was denser in perinuclear regions. The data suggest that the virtual absence of events at the periphery of normal myotubes was not due to a lack of release channels of either isoform.

**DISCUSSION**

This study demonstrates a straightforward topographic segregation of developing muscle cells into central regions, where Ca\(^{2+}\)/H\(\text{11001}\) sparks occur spontaneously, and peripheral regions, comprising ~20% of the radius, where T-tubular structures are present and Ca\(^{2+}\)/H\(\text{11001}\) sparks are largely absent. The study also found evidence that DHPRs in the T tubules appear to have a crucial role in this inhibitory effect.

**T tubule structures are essential for preventing Ca\(^{2+}\)/H\(\text{11001}\) sparks.** Ca\(^{2+}\)/H\(\text{11001}\) sparks of skeletal muscle result from the brief concerted opening of clusters of release channels (23). In mammalian muscle, few Ca\(^{2+}\)/H\(\text{11001}\) sparks were observed in fibers with intact plasmalemma (11). To induce Ca\(^{2+}\)/H\(\text{11001}\) sparks in mammalian muscle, the T tubule membrane had to be permeabilized by application of saponin (27, 66). This suggests that the integrity of the T tubule structure may be necessary to prevent spontaneous opening of release channels. Embryonic myotubes,
which express Ca\textsuperscript{2+} release channels but have an incompletely developed T tubular structure, were used to test this hypothesis. With the use of a double-staining technique, Ca\textsuperscript{2+} sparks and T tubule structures were imaged simultaneously. First, it was demonstrated that the regions that gave rise to sparks were essentially devoid of T tubules. Conversely, the regions that did not produce sparks correspond to areas of the cell that had T tubules. It was also shown that the absence of sparks in the T tubular regions was not due to a lack of expression of RyR isoforms, of either sort.

The present work shows that T tubules and sparks occupy mutually exclusive spaces. As a consequence, the suppression of sparks must be due to T tubule-associated components, or to structures that are modified when the T tubule integrity is affected. At the molecular level, the suppression must be due to an interaction between release channels and junctional structures of some sort. The conclusion is consistent with the rapid decrease in frequency of spontaneous Ca\textsuperscript{2+} sparks in mouse muscle observed in the first two postnatal weeks (8), at a time when development of T tubules is becoming complete (57). It is also consistent with the existence of a malignant hyperthermia-inducing mutation in the III-IV loop of the DHPR in humans (65), which presumably weakens a basal inhibitory effect on RyR channels.

No T-SR docking is required for production of Ca\textsuperscript{2+} sparks. According to Takekura et al. (57), ontogenesis of Ca\textsuperscript{2+} release units requires docking of SR to embryonic plasmalemma or T tubules, to permit local clustering of release channels and voltage sensors. In contrast with this view, the present results demonstrate the presence of functional sources of Ca\textsuperscript{2+}, capable of generating sparks in regions of SR not yet docked to the T tubule network. It follows that clusters of channels capable of activation form at stages before T-SR docking. Therefore, docking does not appear to be necessary for development of a spark-capable cluster. Instead, the effect of linkage with T tubules appears to be to put such clusters under a strong
inhibitory control, which suppresses their ability to fire under normal conditions.

While these “unmoored” regions of SR generate sparks, the events are profoundly different from sparks observed in adult muscle (67). Most notably, their spatial width is nearly threefold greater in myotubes, which is only possible if the activation propagates over large, extensive groups of channels. Again, this is an indication of a susceptibility to activation that is largely lost on docking with T tubules.

**DHPRs are necessary for basal suppression of Ca\(^{2+}\) sparks.** DHPRs in the T tubule membrane function as activators, which open release channels in the SR membrane during an action potential through a direct interaction. Some studies have also shown that DHPRs may function as inhibitors for the Ca\(^{2+}\) release channels, including the work of Suda (55), indicating possible involvement of DHPRs in terminating Ca\(^{2+}\) release in rat skeletal myotubes, blockage of Ca\(^{2+}\) release from SR vesicles by a peptide derived from the II-III loop of the DHPR (13), an effect of DHPR channel blockers on caffeine sensitivity of 1B5 myotubes expressing RyR1 (33) and the demonstration that the R1086H mutation in \(\alpha_{1S}\) enhances sensitivity to activation by the voltage sensor and by caffeine (65).

To test for a role of DHPRs in situ we used mdg myotubes, which form T-SR junctions (20) lacking DHPRs in the T tubule membrane but are structurally normal otherwise. The location of Ca\(^{2+}\) sparks was random in these cells, indicating that T tubules without DHPRs no longer prevented spontaneous opening of the release channels. The DHPR is therefore required for this inhibitory effect.

The test required changes in conditions that made the comparison less clear cut. In dysgenic cells it was necessary to increase \([\text{Ca}^{2+}]_o\) radically and apply 1 mM caffeine to even observe sparks. Complicating matters, when wild-type cells were observed in the high-\([\text{Ca}^{2+}]_o\) solution with caffeine, an even higher frequency of sparks was induced, together with the loss of the characteristic pattern of locations.

In an attempt to compare wild-type and mdg cells under similar conditions of load, the normal myotubes were imaged in high Ca\(^{2+}\), the addition of 50 \(\mu\)M nifedipine to block Ca\(^{2+}\) entry through L-type Ca\(^{2+}\) channels. The similarity in morphological parameters of Ca\(^{2+}\) sparks recorded in mdg and in normal cells under these conditions was an indication that rough parity in cytosolic and intra-SR Ca\(^{2+}\) levels had been reached. The normal myotubes in higher \([\text{Ca}^{2+}]_o\) still maintained the suppression pattern, consistent with a necessary role of DHPRs in the basal inhibition of release channels.

We initially thought that the reason for the absence of sparks in mdg cells could be a lower \([\text{Ca}^{2+}]_c\) due to decreased Ca\(^{2+}\) influx due to the absence of DHPRs (the L-type Ca\(^{2+}\) channels in the plasmalemma), a lower load of Ca\(^{2+}\) in the SR secondary to the diminished influx, or both. Indeed, when \([\text{Ca}^{2+}]_o\) was increased, the dysgenic cells generated sparks at a higher rate, comparable with the wild-type cells in Krebs.

However, direct measurements of \([\text{Ca}^{2+}]_c\) and \([\text{Ca}^{2+}]_s\) in wild-type and mdg cells immersed in Krebs solution did not support this explanation because \([\text{Ca}^{2+}]_c\) was nearly identical in wild-type and mdg cells immersed in Krebs solution. In any case, the final conditions used for comparing patterns of spark location led to similar \([\text{Ca}^{2+}]_c\) and \([\text{Ca}^{2+}]_s\) in both types of cells. The \([\text{Ca}^{2+}]_c\) measurements therefore support an inhibitory role of the DHPR, as they show that the difference in patterns may not be attributed to differences in relevant \([\text{Ca}^{2+}]_o\) under the conditions that made the comparison possible.

**The suppression effect in embryonic cells is conserved in adults.** As first described by Shirokova et al. (53) the topographic segregation between T tubules and Ca\(^{2+}\) sparks has a
two-way functional correlate, which consists of an absence of voltage-operated release in the regions where sparks are observed, plus a pattern of voltage-operated release (where it exists) that is devoid of sparks. The failure of voltage sensors in the mammal to elicit sparks was first shown in adult muscle (52). The picture was recently completed with the finding that the elementary events produced by depolarization in the rat are “embers,” resulting from the opening of single RyR channels (11).

Thus it appears that DHPRs and RyRs engage in a peculiar functional relationship. It includes a basal inhibition of sparks, plus a dynamic, voltage-dependent activation of opening accompanied by continued inhibition of sparks even when channels open. This peculiar feature is also present in myotubes, as shown by Shirokova et al. (53). Therefore, the DHPR-RyR interaction at work in the embryonic cells produces fully the “adult” effect.

The fact that the inhibitory effect of the DHPR on sparks is observed in both adult and immature mammalian muscle cells has intriguing implications. We showed above that the inhibition of sparks associated with T-tubular structures (and presumably the DHPRs) occurs even in regions where RyR3 isoforms are present. The inhibitory effect, which could be attributed to the mechanical interaction between DHPRs and RyR1, somehow is capable of inhibiting the other isoform, which is believed not to be under any sort of mechanical interaction with voltage sensors (49). That the effect of T-tubular structures on RyR1 channels also prevents RyR3 channels from causing sparks is difficult to explain, as RyR3 are capable of producing sparks when expressed alone in 1B5 cells.

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**Fig. 10.** The evolution of SR luminal \(\text{Ca}^{2+}\). A: image \(F_{33}\) of rhod-2 fluorescence, 1 min after being immersed in an \(mdg\) culture dish in permeabilizing solution. B: fluorescence \(F_{32}\) of mag-indo-1. Note bright cell in B, a myotube not yet permeabilized, seen as a dark region in A (dashed contour). C: ratio of images \(F_{31}\) (not shown) and \(F_{32}\). Note lower ratio in the cell that is not permeabilized. D and E: ratio images in wild-type culture dishes, permeabilized after 10 min in Krebs (D) or K/Ca (E). F: evolution of average ratio (and \([\text{Ca}^{2+}]_{SR}\)) vs. time after immersion in permeabilizing solution of the culture in D (black symbols) or E (red).

**Fig. 11.** The effects of extracellular \([\text{Ca}^{2+}]_o\) on \([\text{Ca}^{2+}]_{SR}\). Means \pm SE over all mag-indo-1 SEER images obtained in the same condition. The number of fields included in the averages is listed. Note the near identity of levels attained in wild-type cells in K/Ca solution with nifedipine, and in \(mdg\) cells in K/Ca.
It suggests an intricate interaction, such that the isoform 3 channels cannot engage in coordinated opening unless the RyR1 channels are also enabled.

**DHPRs are not sufficient for basal suppression of Ca\(^{2+}\) sparks.** Although the present studies point at an important role of the DHPRs in the suppression of sparks, they also demonstrate that the suppressive effect can be overcome. This occurs when normal myotubes are exposed to a high \([\text{Ca}^{2+}]_o\). Chun et al. (8) demonstrated an association between “spontaneous” Ca\(^{2+}\) sparks in myotubes and Ca\(^{2+}\) current through L-type Ca\(^{2+}\) channels. The increased unitary current that presumably crosses these channels in conditions of high \([\text{Ca}^{2+}]_o\) appears to be sufficient to overcome the basal inhibitory effect.

A second instance, in which the inhibitory effect is relieved to a degree, is the chemical permeabilization of the plasmalemma by brief exposure to the mild detergent saponin. The interaction between voltage sensors and release channels may be altered upon exposure to saponin (32). Alternatively, Shirokova and coworkers (26) proposed that an alteration of mitochondrial function, occurring progressively after saponin treatment, is what determines the production of sparks and overcomes the inhibition by T tubule structures.

Work on mechanically skinned skeletal muscle fibers supports the alternative. After the plasmalemma is peeled, T tubules reseal, restore their transmembrane potential, and maintain voltage-dependent skeletal type EC coupling (30). This implies that in peeled fibers DHPRs must be in a normal functional and structural interaction with Ca\(^{2+}\) release channels. As shown by Kirsch et al. (27) and confirmed with rat muscle in our laboratory (B. S. Launikonis and E. Rios, unpublished observations), spontaneous sparks occur in these fibers, albeit at a lower rate.

Therefore, the presence of the DHPRs is one of the conditions necessary to prevent the occurrence of Ca\(^{2+}\) sparks in the mammal. It should be noted that the functional state of the DHPR is immaterial for this effect, as the suppression persists in depolarized fibers (hence with voltage-inactivated DHPRs; Refs. 11 and 53) or as shown here, in the presence of a high dose of nifedipine, which drives the DHPRs to the same inactivated state.

**Fig. 12. Immunolocalization of ryanodine receptor (RyR) isoforms in myotubes.** A–C: fluorescence of fluorescein conjugates of wild-type primary myotubes labeled with the anti-RyR1 (A and B) or anti-RyR3 antibody. D–F: corresponding images of dysgenic myotubes. Images reveal a clustered distribution of both RyR1 and RyR3 in the entire cytosolic region of the cells.
supramolecular arrangement (saponin, mechanical peeling, and mitochondrial alteration) or an increase in the inward current through L-type channels are capable of overcoming the functional inhibition.

Measuring $[\text{Ca}^{2+}]_{\text{cyto}}$ with SEER. By increasing the dynamic range of ratiometric measurements, SEER makes it possible to image $[\text{Ca}^{2+}]$ inside organelles, as initially demonstrated using mag-indo-1 (31). Here we used the higher-affinity dye indo-1 to monitor $[\text{Ca}^{2+}]$ in the cytosol. Like other measurements with dyes loaded into the cytosol in AM form, the present one is compromised by entry of dye into organelles. In the Appendix, we introduce an approach to correct such errors, which results in Eq. 2 relating $[\text{Ca}^{2+}]$ to the measured ratio. Equation 2 is isomorphic with the conventional Eq. 1, but has an effective dissociation constant, $K_{\text{eff}}$, smaller than $K_D$, and an effective minimum ratio, $R_0$, greater than $R_{\text{min}}$. This correction is liable to introduce errors, as it assumes the presence of a constant fraction of the dye in the organelles. However, the Appendix shows that the magnitude of the correction is proportional to the combined fractional volume of SR and mitochondria in myotubes, which is probably <10% of the total (C. Franzini-Armstrong, unpublished observations). Therefore, even if the dye load and volume of organelles change from cell to cell, the errors in Eq. 2 will probably stay small.

The normal myotubes studied here had on average a $[\text{Ca}^{2+}]_{\text{cyto}}$ of 152 nM. This value is in good agreement with results obtained in normal myotubes by Shimahara et al. (50) using fura-2, and those measured by Pérez et al. (45) in 1B5 myotubes expressing RyR1 in a careful study with Ca$^{2+}$-sensitive microelectrodes, but greater than values of Avila et al. (1) using a null-point calibration approach in normal myotubes with indo-1. Considering the many sources of error, the agreement among all these approaches appears reasonable.

$[\text{Ca}^{2+}]_{\text{cyto}}$ measured in mdg myotubes was on average 172 nM, which is very close to the value in wild-type cells. Similar concentrations in these two types of cells were also reported by Shimahara et al. (50). While we know of no other comparisons between normal and dysgenic myotubes, Weiss et al. (65) reported that un.injected and DHPR α1s-expressing mdg cells had the same resting indo-1 fluorescence ratios. In all, different approaches lead to the conclusion that lack of the L-type Ca$^{2+}$ channel/voltage sensor per se does not determine a major change in resting cytosolic $[\text{Ca}^{2+}]$.

Cytosolic $[\text{Ca}^{2+}]$ increased significantly in normal myotubes exposed to high $[\text{Ca}^{2+}]_c$. The increase was due in large part to influx through a DHSP-sensitive pathway, as it was nearly abolished by 50 μM nifedipine. In dysgenic cultures however, the exposure to high $[\text{Ca}^{2+}]_c$ did not lead to higher $[\text{Ca}^{2+}]_{\text{cyto}}$. One may conclude from these observations that exposure to a high $[\text{Ca}^{2+}]_c$ increases $[\text{Ca}^{2+}]_{\text{cyto}}$ through an increase in basal entry of Ca$^{2+}$ through L-type Ca$^{2+}$ channels open at rest.

Imaging $[\text{Ca}^{2+}]$ in stores of normal and dysgenic myotubes. The present study includes for the first time confocal ratiometric images of a $[\text{Ca}^{2+}]$-sensitive dye in stores of myotubes. Unlike the images of indo-1 in the cytosol, those of mag-indo-1 in organelles reveal structural detail. As the ratiometric measurement is independent, to a good degree, of local dye concentration and volume of the stained organelle, the local variations in the ratio reflect changes in organellar $[\text{Ca}^{2+}]$.

In adult cells, two types of structures were described, with clearly different SEER ratios: areas of high ratio, corresponding to SR terminal cisternae, and longitudinal structures of low ratio, identified as mitochondria (31). As shown, for example, in Figs. 9 and 10, there is no comparable pattern in myotubes. It is clear that nuclei do not load well with dye. But perinuclear areas sometimes exhibit a different ratio (Figs. 9C and 10C). Perhaps relative development of mitochondria and SR change among different cultures. Given this variability, we have not attempted functional and structural separation of the main organelles in these cells.

The resting $[\text{Ca}^{2+}]$ in stores was 82 μM in normal myotubes. This is substantially lower than in adult frog muscle, where the same technique yields an average of 470 μM for permeabilized fibers equilibrated in a solution with 100 nM $[\text{Ca}^{2+}]_{\text{cyto}}$ (31). The measurement in myotubes may have been distorted by a contribution from mitochondria. In addition, the concentration of intra-organellar dye was two- to fourfold lower in myotubes than in adult cells, which may have biased the ratio toward lower values. In conclusion, the present measurements, which indicate a major difference in SR load between embryonic mouse and adult frog cells, may have underestimated $[\text{Ca}^{2+}]_{\text{SR}}$ to some extent.

The measurement, however, should provide a good comparison tool. Thus, it demonstrated an increase in organellar $[\text{Ca}^{2+}]$, partially prevented by nifedipine, upon exposing normal myotubes to elevated $[\text{Ca}^{2+}]_c$. This result is consistent with the observation of higher $[\text{Ca}^{2+}]_{\text{cyto}}$ under the same conditions (Fig. 8). The fact that the increase in $[\text{Ca}^{2+}]_{\text{cyto}}$ took many minutes, even in $[\text{Ca}^{2+}]_c$ as high as 26 mM, is consistent with the observation that Ca$^{2+}$ load in stores of embryonic muscle fibers did not change measurably after 10 min of exposure to 8 mM $[\text{Ca}^{2+}]_c$ (8).

$[\text{Ca}^{2+}]_{\text{SR}}$ was expected to be close to normal in mdg cells, as many structure-function studies have demonstrated (and relied upon) the integrity of the Ca$^{2+}$ store in these cells. Surprisingly, intra-organellar $[\text{Ca}^{2+}]$ was significantly greater in the dysgenic cells, by about twofold. The present study is not the first to imply greater load in dysgenic cells; Weiss et al. (65) found that the maximal cytosolic Ca$^{2+}$ transient in response to caffeine was greater in uninjected than in α1s-expressing dysgenic cells, by nearly 40%. As we have shown, dysgenic cells do not produce spontaneous Ca$^{2+}$ sparks in Krebs, but normal myotubes do. The difference in $[\text{Ca}^{2+}]_{\text{SR}}$ could be in part due to the greater leak constituted by large and frequent release events in normal cells at rest.

This explanation may help justify the paradoxical effect of exposure to high $[\text{Ca}^{2+}]_c$ in dysgenic cells: a decrease in $[\text{Ca}^{2+}]_{\text{SR}}$. As shown previously, elevated $[\text{Ca}^{2+}]_c$ does not significantly increase $[\text{Ca}^{2+}]_{\text{cyto}}$ in these cells, perhaps because their only pathways of Ca$^{2+}$ entry may be SOC or ECCE channels (7, 44), with currents that saturate at relatively low $[\text{Ca}^{2+}]_c$ (7). High $[\text{Ca}^{2+}]_c$ causes sparks to appear, providing a leak that may shift the steady $[\text{Ca}^{2+}]_{\text{SR}}$ to a lower value. However, these explanations are incomplete, as they do not provide a reason for the absence of Ca$^{2+}$ sparks in mdg myotubes at rest, or their appearance in high $[\text{Ca}^{2+}]_c$.

The conditions found appropriate for comparing the patterns of spark generation in normal and dysgenic myotubes included high $[\text{Ca}^{2+}]_c$, 1 mM caffeine, and, in normal myotubes, 50 μM nifedipine. Under those conditions, free $[\text{Ca}^{2+}]_{\text{cyto}}$ was 192 nM.
in the wild-type cells and 118 nM in the dysgenic cells (Fig. 8). In similar solutions, organellar [Ca^{2+}] was 144 µM in both types of cells (Fig. 11). Given these results, the characteristic spatial location of Ca^{2+} sparks in the wild type is likely to be due to differences in modulation by Ca^{2+}. It may reflect instead a direct inhibitory effect of the DHPR, missing in dysgenic cells.

**APPENDIX**

Ratiometric measurements of [Ca^{2+}]_{Cys} corrected for dye inside organelles. Like other ratiometric measurements with dyes loaded into cells in AM form, the SEER measurement introduced in the text is affected by errors due to entry of dye into organelles. The following is an approach to correct for such errors.

Organelles occupy a certain fraction of the cell volume, and load dye, free or bound, at concentrations that may or may not be equal to that in the cytosol. Let \( v \) represent the fraction of the dye load present in organelles (equal to the fractional volume where the organellar dye would partition, if its concentration was the same in organelles and cytosol). Assuming that luminal [Ca^{2+}] is sufficient to saturate the dye, fluorescence \( F_{11} \) will satisfy

\[
F_{11} = v f_{11,Ca} + \frac{(1 - v)f_{11}[Ca^{2+}] + f_1 K_D}{[Ca^{2+}] + K_D}
\]

where \( f_{11} \) and \( f_{11,Ca} \) are, respectively, the fluorescence per unit concentration of free and Ca^{2+}-bound dye. An analogous expression applies for \( F_{22} \). Solving for [Ca^{2+}] the definition equation \( R = F_{11}/F_{22} \), it follows that

\[
[Ca^{2+}] = K_D \beta (1 - v) \frac{(\beta (1 - v) + v) R - \beta (1 - v) R_{min} - v R_{max}}{(1 - v) + v (R_{max} - R)}
\]

where \( \beta \) is \( f_{22}/f_{22}\text{Ca} \), \( R_{max} \) is \( f_{11,Ca}/f_{22,Ca} \), and \( R_{min} \) is \( f_{11}/f_{22} \).

This is the equation of Grynkiewicz et al. (25) (Eq. 1 in methods), modified to correct for the interference of a dye in organelles. It can be shown that Eqs. 4 and 1 are isomorphic, by defining

\[
K_{eff} = K_D (1 - v)
\]

and

\[
R_0 = \frac{\beta (1 - v) R_{max} + v R_{max}}{\beta (1 - v) + v}
\]

Equation 2, repeated here, is obtained substituting these definitions in Eq. 4:

\[
[Ca^{2+}] = K_{eff} \beta \frac{R - R_0}{R_{max} - R}
\]

The interference from dye in organelles therefore results in a change in \( K_D \) to a value smaller than \( K_D \), and an effective minimum ratio, to a value greater than \( R_{min} \) \( R_0 \) is an average of \( R_{min} \) and \( R_{max} \) with weights \( \beta (1 - v) \) and \( v \). The modified parameters, determined in an on-cell calibration, gave \( K_{eff} = 394 \text{ nM} \) and \( R_0 = 0.51 \).

The limitations of this correction should be obvious: it assumes the presence of a constant fraction of the dye in the organelles, and it assumes, perhaps more safely given the high affinity of indo-1, that [Ca^{2+}] there is sufficient to saturate the dye. While the first assumption is unlikely to hold as more than a rough approximation, the correction is small. The combined volume of SR and mitochondria in myotubes is probably <10% of the total (C. Franzini-Armstrong, unpublished observations). If \( v \) is 0.1, then \( K_{eff} \) will be 0.9 \( K_D \) and \( R_0 \) will be very close to \( R_{min} \) (~30 times closer to \( R_{min} \) than \( R_{max} \) according to Eq. 6). Therefore, even if the dye load and volume of organelles change from cell to cell, the errors in Eq. 7 (see Eq. 2) will probably remain low.

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**REFERENCES**


REPRESSION OF CA2+ SPARKS BY T TUBULES