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Ca^{2+} influx through α_{1S} DHPR may play a role in regulating Ca^{2+} release from RyR1 in skeletal muscle

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Shtifman, Alexander, Cecilia Paolini, José R. López, Paul D. Allen, and Feliciano Protasi. Ca^{2+} influx through α_{1S} DHPR may play a role in regulating Ca^{2+} release from RyR1 in skeletal muscle. *Am J Physiol Cell Physiol* 286: C73–C78, 2004. First published September 3, 2003; 10.1152/ajpcell.00194.2003.—Differentiated primary myotubes isolated from wild-type mice exhibit ryanodine-sensitive, spontaneous global Ca^{2+} oscillations as well as spontaneous depolarizations in the plasma membrane. Immunolabeling of these myotubes showed expression of both α_{1S} dihydropyridine receptors (DHPRs) and ryanodine-sensitive Ca^{2+}-release channel 1 (RyR1), the two key proteins in skeletal excitation-contraction (E-C) coupling. Spontaneous global Ca^{2+} oscillations could be inhibited by addition of 0.1 mM $CdCl_2$/0.5 mM $LaCl_3$ or 5 μM nifedipine to the extracellular bathing solution. After either treatment, Ca^{2+} oscillations could be restored upon extensive washing. Although exposure to DHPR antagonists completely blocked Ca^{2+} oscillations, normal orthograde signaling between DHPRs and RyRs, such as that elicited by 80 mM KCl depolarization, was still observed. In addition, we showed that spontaneous Ca^{2+} oscillations were never present in cultured mdg myotubes, which lack the expression of α_{1S}DHPRs. These results suggest that under physiological conditions in conjunction with the mechanical coupling between the α_{1S}DHPRs and RyR1, the initiation of Ca^{2+} oscillations in myotubes may be facilitated, in part, by the Ca^{2+} influx through the α_{1s}-subunit of the DHPR.

calcium-induced calcium release; dihydropyridine receptors; excitation-contraction coupling; ryanodine receptors; skeletal muscle

EXCITATION-CONTRACTION (E-C) coupling in muscle involves a rapid cascade of events that transform the surface membrane depolarization into Ca^{2+} release and generation of force. This sequence of events is initiated by an action potential that upon reaching the transverse tubules (TT) activates the L-type Ca^{2+} channels, the dihydropyridine receptors (DHPRs). Activation of the DHPRs triggers a sudden release of Ca^{2+} from the sarcoplasmic reticulum (SR) via Ca^{2+} release channels (ryanodine receptors, RyRs) that reside in the junctional region of the SR immediately adjacent to the TT membrane. The resulting release of Ca^{2+} produces a transient increase in intracellular [Ca^{2+}], which activates the contractile apparatus of muscle fibers (for review, see Ref. 20). Although the major components of the signal transduction between the two membrane systems have been identified, the precise mechanisms that govern this process are not fully resolved.

DHPRs and RyRs are the two primary proteins involved in the E-C coupling in skeletal, cardiac, and smooth muscle. However, it has become increasingly evident that the communication between these two proteins in skeletal muscle employs a different mechanism compared with the cardiac and smooth muscle. In cardiac and smooth muscle, activation of the α_1DHPR generates a large inward flux of Ca^{2+} from the extracellular space, which induces the opening of RyRs and a consequent massive release of Ca^{2+} into the myoplasm. This mechanism has been defined as calcium-induced calcium release, or CICR (6). In skeletal muscle, however, initiation of a contraction can be achieved in the absence of the extracellular Ca^{2+} (3, 4). In fact, it has been generally accepted that in skeletal muscle α_{1S}DHPR functions predominantly as the voltage sensor (24, 30) that activates the RyRs though a physical interaction, known as the orthograde signaling. Although the role of Ca^{2+} influx in skeletal muscle E-C coupling is unclear, Ca^{2+} ions do participate in modulation of the RyR activity through a process similar to that of cardiac CICR (5a, 17–19).

In the present work, we report the existence of spontaneous, RyR-mediated Ca^{2+} transients, as well as spontaneous depolarizations of the plasma membrane in primary myotubes of skeletal origin. The ability of Ca^{2+} channel blockers to eliminate these events indicates that the repetitive pattern of this activity is dependent upon the influx of Ca^{2+} through the DHPRs and suggests that Ca^{2+} current through α_{1S}DHPRs may play a relevant role in developing skeletal muscle cells.

MATERIALS AND METHODS

Cell culturing. Forelimb and hindlimb muscles were removed from wild-type and dysgenic neonatal mice. Satellite cells were selected as described elsewhere (28). Briefly, cells were enzymatically dissociated from minced muscle by the addition of a dispase solution (2 ml/g of tissue, grade II, 2.4 U/ml; Boehringer Mannheim, Indianapolis, IN) and collagenase (class II, 1%; Boehringer Mannheim) supplemented with $CaCl_2$ to a final concentration of 2.5 mM. The cell slurry was maintained at 37°C for 30–45 min, triturated every 15 min with a 5-ml plastic pipette, and filtered through 80 μm of nylon mesh (NITEX; Tetko, Monterey Park, CA). The filtrate was spun at 350 *g* to sediment the dissociated cells. The obtained pellet was resuspended in growth medium, and the suspension was plated on collagen-coated dishes. The cell culture was expanded on enhanced chemiluminescence (ECL) coated dishes at 37°C in a growth medium composed of low-glucose Dulbecco's modified Eagle's medium (DMEM)

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(GIBCO, Invitrogen, Grand Island, NY), supplemented with 20% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, and 20 mM basic fibroblast growth factor (bFGF) (Promega, Madison, WI). After ~36/48 h, the cells were detached and plated on either *1*) 35-mm dishes containing thermanox coverslips for immunocytochemistry (Nunc, Naperville, IL) or *2*) 96-well plates with ultrathin, clear bottoms (Corning, Costar, NY) coated with Matrigel (BD Bioscience, Bedford, MA). When cells reached ~40% confluence, the growth medium was replaced with differentiation medium that was composed of DMEM supplemented with 5% heat-inactivated horse serum, 1% L-glutamine, 1% penicillin, and 1% streptomycin. All media were changed daily.

Immunohistochemistry. The cells were fixed in methanol for a minimum of 20 min at −20°C. To avoid nonspecific detection, cells were blocked for 1 h in PBS supplemented with 1% BSA and 10% goat serum. Cells were incubated at room temperature with the appropriate primary antibody for 2 h and then washed three times for 10 min with PBS/BSA before being incubated for 1 h with secondary antibodies. Code, specificity, working dilution, and the sources of primary antibodies used in single staining experiments are as follows: anti-RyR 34C, 1:30 (2) (Developmental Studies Hybridoma Bank, The University of Iowa); sheep anti-α_{1S}DHPR, 1:500, Upstate Biotechnology, Lake Placid, NY. Secondary antibodies were conjugated with either cyanine 3 or cyanine 5 (Jackson ImmunoResearch Laboratories, Lexington, KY). The specimens were viewed on a laser scanning confocal microscope (Zeiss LSM510, Specifics) interfaced with an inverted Zeiss Axiovert microscope.

Fluorescence measurements. Intracellular Ca^{2+} imaging was performed as described previously (22, 26). Briefly, the differentiation media were removed and cells were washed twice with imaging buffer (IB) containing 125 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 6 mM glucose, 25 mM HEPES, 0.05% BSA, 2 mM $CaCl_2$, pH 7.4 (for those conditions where depolarization of the myotubes was required, IB contained 50 mM NaCl and 80 mM KCl). Cells were then loaded for 30 min with Ca^{2+} indicator dye (fluo 4-AM, 10 μM) and washed several times with IB to terminate further loading. Whole cell fluorescence changes were detected using PTI delta-RAM as the light source with a 12-bit digital intensified charge-coupled device (Stanford Photonics) interfaced with an inverted microscope equipped with an Olympus Uapo/340 ×40 oil immersion objective. Changes in intracellular Ca^{2+} were characterized as changes in fluo 4 fluorescence intensity. All experiments were conducted at room temperature (22°C). Solution exchange within each well was achieved via pressure controlled perfusion system (Automate Scientific, Berkley, CA). The perfusion inlet was positioned close to the cells to allow a very efficient and rapid change of solution. Detected changes in fluorescence from the regions of interest within each cell were analyzed using QED imaging software (QED Software, Pittsburgh, PA). The resulting fluorescence changes were corrected for the background fluorescence within individual cells by dividing the value of the fluorescence intensity at each measured interval by the mean fluorescence intensity of a 30-s quiescent period within that cell to give the F/F_0 values.

Microelectrode preparation and membrane potential recording. Microelectrodes used in the recording of the membrane potential were prepared from thin-walled 1.5/1.12 mm internal diameter borosilicate glass capillaries with internal filaments (WPI-TW150-4). Before pulling, the capillaries were washed with 1 M HCl and distilled water and dried at 150°C for 3 h. The clean glass capillaries were drawn into microelectrodes by using a Flaming Brown puller model P-87 (Sutter Instruments, San Francisco, CA). The microelectrodes were backfilled with filtered 3 M KCl immediately before use and had a tip resistance ranging from 10 to 15 MΩ. The bath reference electrode was an Ag-AgCl pellet.

Single myotubes were carefully impaled with the aid of an inverted compound microscope (Axiovert 10) fitted with a ×10 eyepiece and a ×40 dry objective. The potential from the 3 M KCl barrel (V_m) was recorded with a WPI high-impedance amplifier F-223A (Sarasota, FL). The V_m potential was filtered at 5–10 KHz to improve the signal to noise ratio and was stored for further analysis. All membrane potential recordings were carried out at 22°C.

RESULTS

RyR-mediated spontaneous activity in skeletal myotubes. We have observed that cultured primary myotubes isolated from mouse limb muscle exhibit spontaneous, regenerative macroscopic Ca^{2+} release activity (Fig. 1*A*). The frequency of appearance of this phenomenon increased with the duration of time that the cells were kept in differentiation media, but it was always present in at least 25–30% of the cells in each culture. Changes in intracellular Ca^{2+} were detected as changes in the fluorescence intensity of a Ca^{2+} indicator, fluo 4-AM. These spontaneous events had a distinctly different morphology than the Ca^{2+} transients elicited by either KCl depolarization (Fig. 1*B*) or caffeine stimulation (not shown). One of the major differences between these spontaneous events and the KCl-induced Ca^{2+} transients is that application of KCl generates a depolarization that sets the membrane potential to a prolonged, stable level that is dependent on the extracellular KCl concentration ($[KCl]_o$), whereas events reported here are initiated by brief, spontaneous depolarizations and repolarizations of the plasma membrane (see Fig. 3). The apparent frequency of regeneration and the amplitude of the oscillations differed among the cells but appeared to be fairly consistent within each individual cell. This activity was also distinctly different from spontaneously occurring Ca^{2+} waves. Whereas Ca^{2+} waves typically originate either within the central region of the cell and slowly propagate outward or initiate at the outer edges of the myotubes and migrate toward the center of the cell, this type of activity appeared as uniform oscillations in fluorescence throughout the entire cell body.

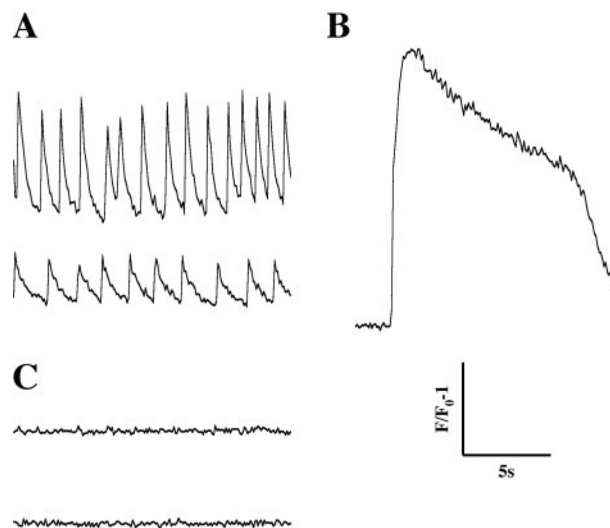

Fig. 1. Ryanodine receptor (RyR)-mediated spontaneous oscillations in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in primary myotubes. *A*: representative fluorescent time courses of primary myotubes exhibiting spontaneous oscillations in $[Ca^{2+}]_i$. (n = 82 cells). *B*: representative response of primary myotubes to KCl-induced depolarization. Cells were depolarized by exposure to 80 mM KCl. *C*: representative fluorescent time courses of primary myotubes treated with 0.5 mM ryanodine (n = 21 cells). Horizontal bar represents time (s), and the vertical bar represents F/F_0 fluorescence intensity (arbitrary units, au).

To determine whether the spontaneous activity was generated by Ca^{2+} release through the RyRs, the myotubes were incubated with 0.5 mM ryanodine for 30 min at 37°C. Ryanodine is a plant alkaloid that binds specifically to the open state of the RyRs and at concentrations above 10 μM locks the channel in a conformation that does not allow for the release of Ca^{2+} (35). As demonstrated in Fig. 1*C*, application of the ryanodine resulted in complete elimination of all detectable Ca^{2+} release activity, suggesting that the observed oscillations in Ca^{2+} occurred as a direct result of the opening of the RyRs.

Expression of key E-C coupling proteins and formation of Ca^{2+} release units. It has been previously reported that immunolabeling of either RyR1 or α_{1S}DHPRs in skeletal muscle myotubes results in a characteristic punctate pattern localized at the periphery of the cell. This pattern indicates clustering of the RyRs and DHPRs and corresponds to the formation of junctions, or calcium release units (CRUs), between SR and exterior membranes in developing myotubes (10, 25, 27). Colocalization of α_{1S}DHPRs and RyR1s is also an indication of correct assembly of skeletal CRUs (10, 27). As demonstrated in Fig. 2, *A* and *B*, wild-type myotubes exhibit a punctate pattern of fluorescence when immunolabeled with either anti-RyR1 or anti-α_{1S}DHPRs antibodies, respectively. The two proteins are not only clustered in bright foci but are also colocalized as demonstrated in Fig. 2*C*, indicating the formation of functional CRUs.

Spontaneous depolarizations in myotubes. As demonstrated in Fig. 1, the observed spontaneous Ca^{2+} oscillations generally appeared at the same frequency and amplitude within each given cell. The uniformity of the release suggests that this activity might be controlled in part by changes in the plasma membrane potential. We have observed that in addition to the cells exhibiting stable resting membrane potential, a subset of cells (~30%) exhibited spontaneous fluctuations in the membrane potential. Figure 3, *A* and *B*, shows membrane potential recordings from a myotube that does not exhibit spontaneous fluctuations in the membrane potential, and from one that does, respectively. Consistent with previous reports (13, 15, 34), the average value of the resting membrane potential was -62 ± 0.44 mV ($n = 48$). The recorded oscillations in the membrane potentials did not appear to resemble action potentials, because the magnitude of the depolarization, 26 ± 1 mV ($n = 146$), was insufficient to reach the action potential threshold. However, on the basis of previous reports, these depolarizations should be sufficient to activate Ca^{2+} influx through the L-type Ca^{2+} channels (32). The percentage of cells tested in each culture exhibiting spontaneous membrane depolarizations correlated with the percentage of cells that exhibited spontaneous intracellular Ca^{2+} oscillations as described in Fig. 1. Analogous to the changes in the intracellular Ca^{2+}, the spontaneous depolarizations occurred without any stimulation of the cells.

Role of DHPR and Ca^{2+} influx in spontaneous oscillations. Because membrane depolarizations activate the voltage sensors in the surface membrane/TT, we sought to determine whether DHPR is directly involved in eliciting these events. To do so, we applied 5 μM nifedipine to the extracellular bathing solution. Nifedipine is a DHP-specific antagonist, which promotes the inactivation of the channel (21, 29) and has a blocking effect on the Ca^{2+} current through the TT membrane (21). As demonstrated in Fig. 4, application of nifedipine abolished the spontaneous RyR-mediated Ca^{2+} release. However, nifedipine could not abolish the KCl-induced Ca^{2+} transients, which

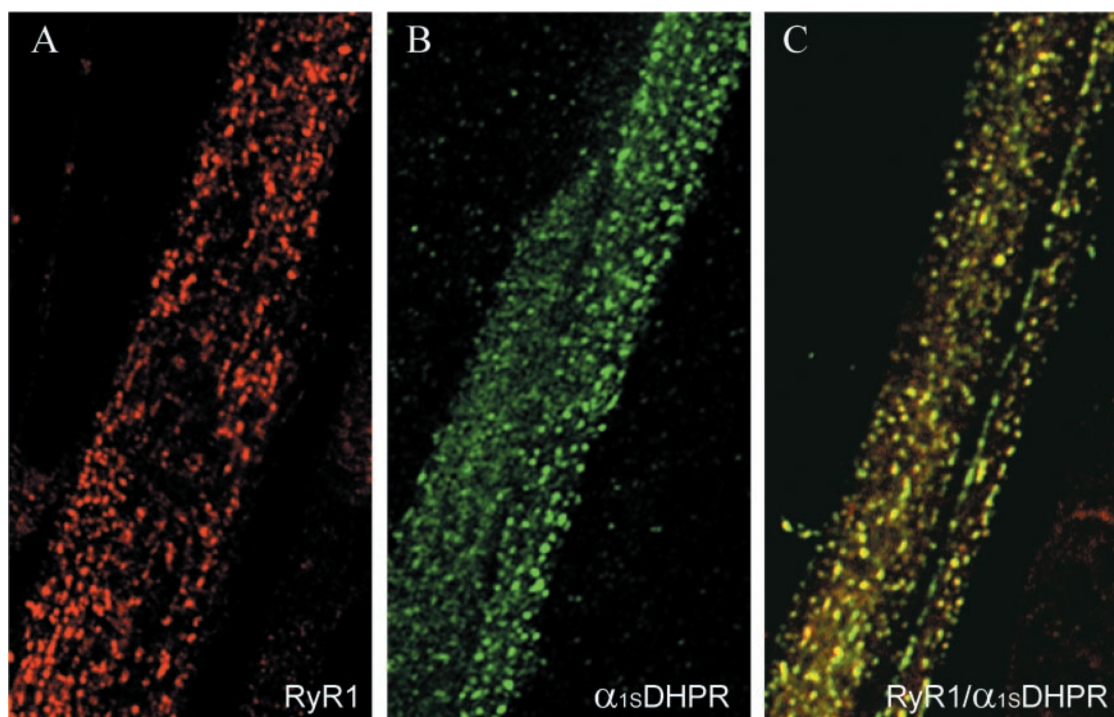

Fig. 2. RyR1 and α_{1S}DHPR (dihydropyridine receptor) are clustered in foci and colocalized in correspondence of calcium release units. *A* and *B*: both RyR1 and α_{1S}DHPR are clustered in discrete foci indicating the targeting of both proteins to junctions between sarcoplasmic reticulum and external membranes. *C*: double immunolabeling for RyR1 and α_{1S}DHPR shows a striking colocalization of the 2 proteins. Bar, 25 μm.

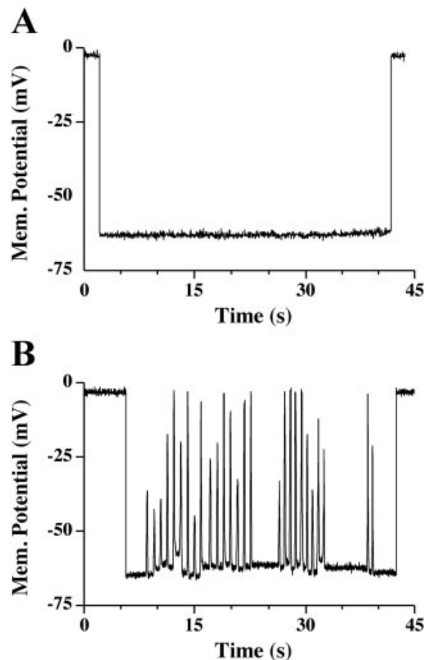

Fig. 3. Primary myotubes exhibit spontaneous membrane depolarizations. *A*: representative recording of resting membrane potential from myotube that does not exhibit spontaneous depolarizations (n = 49 cells). The initial portion of the trace was recorded before myotube impalement; therefore, the membrane potential was adjusted arbitrarily to 0 mV. Impalement of the cell was accompanied by a downward voltage deflection, and the withdrawal of the electrode was reflected by an immediate upward deflection in voltage. *B*: representative recording of a membrane potential from a myotube that exhibits spontaneous membrane depolarizations (n = 10 cells).

could still be elicited in these cells. Further evidence for the involvement of the DHPRs in the spontaneous activity was obtained from experiments conducted with muscular dysgenesis myotubes (mdg), which do not express any functional DHPRs (16, 23). This phenotype renders DHPRs in these cells unable to conduct Ca^{2+} (1) or to participate in E-C coupling (31). None of the tested mdg myotubes exhibited any type of spontaneous activity (Fig. 5). To determine whether these cells expressed functional RyR Ca^{2+} release channels, mdg cells were challenged with 40 mM caffeine. All tested cells produced a robust Ca^{2+} transient in response to the caffeine, indicating a sufficient expression of the RyRs and the viable status of the cells. From these results, it could be inferred that although Ca^{2+} oscillations occurred without any external stimuli, they were under control of the DHPRs.

Ca^{2+} influx through DHPR. To confirm that the abolition of spontaneous activity by nifedipine was based on its inhibition of Ca^{2+} influx and not due to a conformational inactivation of the DHPR, we tested the effects of Cd^{2+} and La^{3+}, which have been previously described as potent blockers of the α_{1S}DHPR channel pore (12, 31). As shown in Fig. 6, application of Cd^{2+}/La^{3+} completely blocked the spontaneous Ca^{2+} oscillations and did so in every cell that exhibited this phenomenon. Washing the cells with the Cd^{2+}/La^{3+}-free solution could reverse the Cd^{2+}/La^{3+} effects. In most cases, the spontaneous activity returned in those cells that exhibited this activity before the application of the Cd^{2+}/La^{3+}. The presence of Cd^{2+}/La^{3+} did not interfere with the large Ca^{2+} transient elicited by depolarization, showing that similarly to nifedipine, Cd^{2+}/La^{3+} blocked spontaneous activity but not the coupling between α_{1S}DHPRs and RyR1.

DISCUSSION

In this study, we report the existence of the spontaneously occurring, regenerative, macroscopic Ca^{2+} transients, as well as spontaneously occurring membrane depolarizations in cultured wild-type myotubes isolated from mouse skeletal muscle. The observed spontaneous activity is generated by the Ca^{2+} release from the RyRs and, most importantly, appears to be triggered by the entry of extracellular Ca^{2+} through α_{1S}DHPRs.

The spontaneous Ca^{2+} transients were observed in a subpopulation of differentiated myotubes and depending on the relative level of cellular differentiation, constituted ~30 to 50% of the entire cell culture. The difference in differentiation rate is due to a number of factors including distribution of myoblasts on the culture plate and regional differences in the concentrations of excreted growth factors. These global oscillations in intracellular Ca^{2+}, although exhibiting variable frequency and amplitude between the cells, were not initiated by any type of applied stimulus and were always significantly smaller then the Ca^{2+} transients detected upon cellular depolarization with KCl. Similar spontaneous Ca^{2+} transients have been previously reported in the cultured human (33) and chicken myotubes (9) and C_2C_{12} mouse cell line (9), as well as embryonic *Xenopus* myocytes (8). However, one of the major differences from previous reports is that the events reported here appear to require the influx of extracellular Ca^{2+}, seemingly through the DHPRs.

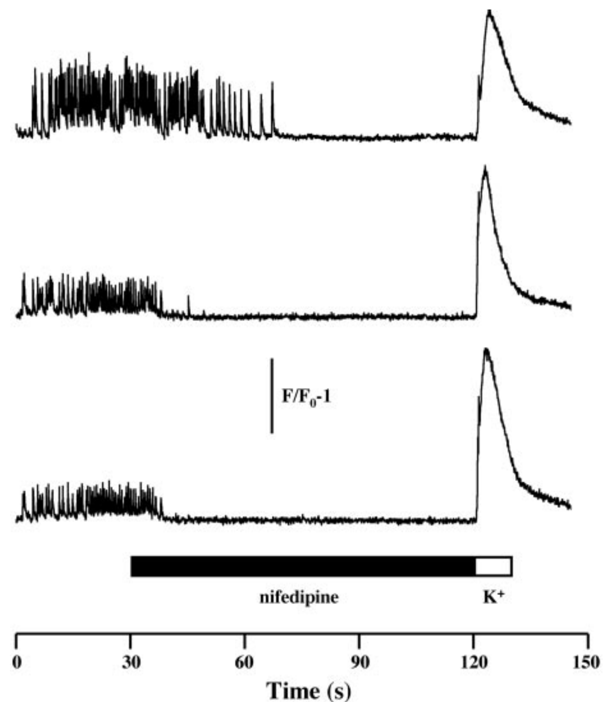

Fig. 4. Inhibition of spontaneous activity in response to nifedipine. Representative, normalized time courses of fluo 4 fluorescence of primary myotubes in response to 5 mM nifedipine and 5 mM nifedipine-supplemented imaging buffer (IB) containing 50 mM NaCl and 80 mM KCl (see MATERIALS AND METHODS for details). Cells were initially perfused for 30 s with IB, followed by a 90-s perfusion with nifedipine (filled bar) and a 10-s stimulation with nifedipine/KCl solution (open bar) (n = 37 cells). The vertical bar represents F/F_0 fluorescence intensity (au).

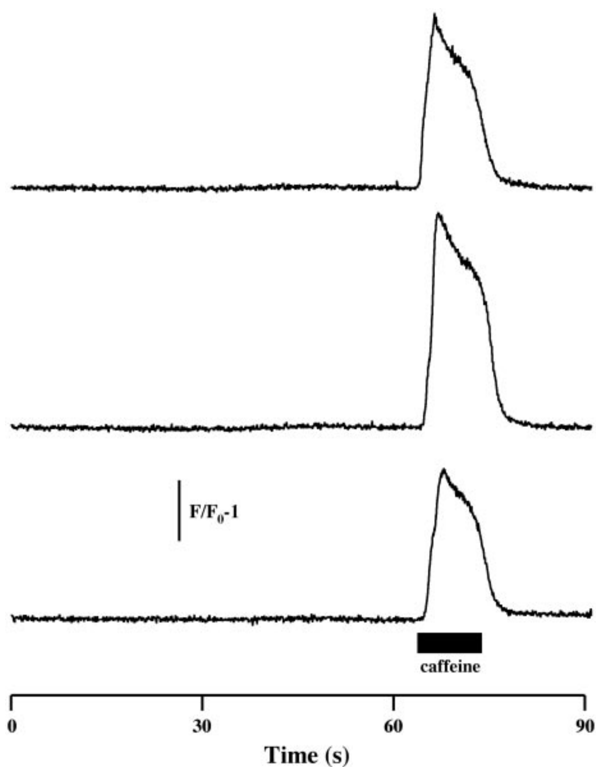

Fig. 5. Dyspedic (*mdg*) myotubes do not exhibit spontaneous activity. Representative, normalized time courses of fluo 4 fluorescence of mdg myotubes in response to 40 mM caffeine. Cells were initially perfused with IB, followed by a 10-s perfusion with caffeine (filled bar) (n = 59 cells). The vertical bar represents F/F_0 fluorescence intensity (au).

There are several lines of evidence suggesting that this type of activity is not simply random Ca^{2+} release from the SR. The fact that the release was uniform throughout the cell and that the oscillations appeared at fixed intervals and with fairly constant amplitudes suggests that there are specific cellular factors that govern the initiation of each oscillation. Results in Figs. 4 and 6 show that by blocking Ca^{2+} influx into the cells either by nonspecific cation channel blockers, such as Cd^{2+} and La^{3+}, or more selectively by nifedipine, these oscillations could be completely inhibited. The fact that application of either of these blockers does not eliminate the depolarization-elicited Ca^{2+} transients, that is, the functional components of the skeletal E-C coupling were still preserved, suggests that the only cause for the change is the abolition of the DHPR Ca^{2+} current. And the fact that intracellular Ca^{2+} oscillations reappear upon the washout of the blockers that restrict the flow of Ca^{2+} suggests that the Ca^{2+} influx is a necessary component of the initiation of this type of activity.

We also demonstrate that a population of myotubes exhibits spontaneous depolarizations of the plasma membrane. It should be pointed out that the observed depolarizations did not resemble typical action potentials. Because the subthreshold depolarizations were not of sufficient magnitude (~35 mV) to reach the threshold of initiation of an action potential, the observed depolarizations could therefore not possibly elicit action potentials. However, these depolarizations must be large enough to activate L-type Ca^{2+} channels to a level sufficient to generate a significant Ca^{2+} influx. Although the membrane potential recordings were performed independently of the intracellular Ca^{2+} measurements, we believe that because the frequency of occurrence of these two observations was similar in both preparations, the two phenomena are related to the same process. If the spontaneous depolarizations precede the activation of Ca^{2+} release from the SR, then it is possible that they activate the voltage sensors in the TT and, subsequently, the DHPR Ca^{2+} channels.

It has been generally accepted that skeletal muscle, unlike cardiac or smooth muscle, does not require the influx of extracellular Ca^{2+} to achieve contraction. One of the reasons it is believed that the influx of Ca^{2+} is simply a vestigial process is because the kinetics of activation are too slow and the magnitude of the current is simply too small for Ca^{2+} to diffuse rapidly from the DHPR Ca^{2+} channel and overcome the Mg^{2+} inhibition of the RyR. To achieve Ca^{2+} influx-induced Ca^{2+} release in skeletal muscle, the skeletal DHPRs would have to behave similar to those of cardiac type with respect to the magnitude and the kinetics of the Ca^{2+} influx. This condition could be potentially achieved in the skeletal muscle if the cells were stimulated by repetitive depolarization (7, 11), analogous to those exhibited in Fig. 3. It has been previously reported that repetitive depolarizations of skeletal muscle fibers at short intervals, as infrequently as 1.7 Hz, result in acceleration, as well as in the potentiation, of the Ca^{2+} currents (7, 11).

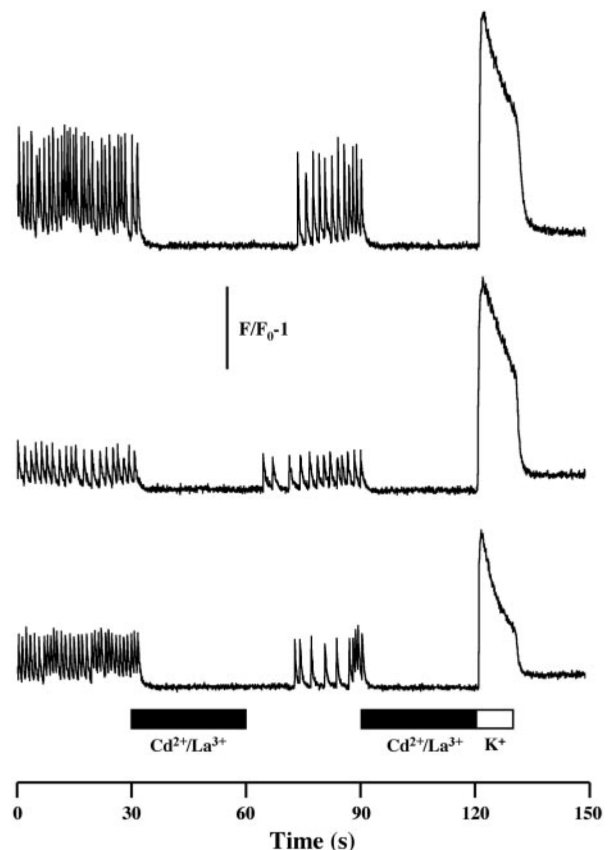

Fig. 6. Cd^{2+}/La^{3+} abolish spontaneous activity. Representative, normalized time courses of fluo 4 fluorescence of primary myotubes in response to 0.5 mM Cd^{2+} and 0.1 mM La^{3+} (filled bar) and 0.5 mM Cd^{2+} and 0.1 mM La^{3+}-supplemented IB containing 50 mM NaCl and 80 mM KCl (see MATERIALS AND METHODS for details) (open bar) (n = 45 cells). Cells were continuously perfused with IB. Where indicated by the bars, IB was supplemented with the appropriate reagents. The vertical bar represents F/F_0 fluorescence intensity (au).

Additionally, it has now been suggested that the magnitude of Ca^{2+} influx through the skeletal DHPR could be sufficient to induce CICR (14). Thus it is conceivable that the initiation of Ca^{2+} release within each oscillation could be triggered by the Ca^{2+} entry through the α_{1s} of DHPRs (5).

In summary, skeletal myotubes exhibit spontaneous oscillations in intracellular Ca^{2+}, as well as spontaneous depolarizations of the plasma membrane. Pharmacological data indicate that initiation of Ca^{2+} oscillations in developing skeletal muscle cells is dependent on the Ca^{2+} influx through the α_{1s}-subunit of the DHPR.

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