Calcium Transients in 1B5 Myotubes Lacking Ryanodine Receptors Are Related to Inositol Trisphosphate Receptors*

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Potassium depolarization of skeletal myotubes evokes slow calcium waves that are unrelated to contraction and involve the cell nucleus (Jaimovich, E., Reyes, R., Liberona, J. L., and Powell, J. A. (2000) Am. J. Physiol. 278, C998–C1010). Studies were done in both the 1B5 (Ry53/−/−) murine “dyspedic” myoblast cell line, which does not express any ryanodine receptor isoforms (Moore, R. A., Nguyen, H., Galceran, J., Pessah, I. N., and Allen, P. D. (1998) J. Cell Biol. 140, 843–851), and C2C12 cells, a myoblast cell line that expresses all three isoforms. Although 1B5 cells lack ryanodine binding, they bind initiated inositol (1,4,5)-trisphosphate. Both type 1 and type 3 inositol trisphosphate receptors were immuno-located in the nuclei of both cell types and were visualized by Western blot analysis. After stimulation with 47 mM K+, inositol trisphosphate mass raised transiently in both cell types. Both fast calcium increase and slow propagated calcium signals were seen in C2C12 myotubes. However, 1B5 myotubes (as well as ryanodine-treated C2C12 myotubes) displayed only a long-lasting, non-propagating calcium increase, particularly evident in the nuclei. Calcium signals in 1B5 myotubes were almost completely blocked by inhibitors of the inositol trisphosphate pathway: U73122, 2-aminoethoxydiphenyl borate, or xestospongin C. Results support the hypothesis that inositol trisphosphate mediates slow calcium signals in muscle cell ryanodine receptors, having a role in their time course and propagation.

Depolarization-induced calcium release in skeletal muscle is generally detected as a fast process mediated by dihydropyridine receptors in the T-tubule membrane and ryanodine receptors (RyR) in the sarcoplasmic reticulum (1–4). We previously found evidence for at least two identifiable calcium signals (5, 6) in skeletal muscle cells in cultures exposed to high external potassium, which suggests that there are at least two calcium release systems that respond to the depolarization signal. The two signals were called the fast and slow waves. We were also able to observe fluorescence heterogeneity during slow waves, corresponding to two separate processes: first, a more rapid increase in fluorescence (the “slow-rapid” wave) that propagates through both nuclei and cytosol regions; second, a slower component of increased fluorescence (the “slow-slow” wave), which is seen only in the nuclear region.

The fact that the cell does not contract during either part of the slow wave indicates that the overall cytosolic calcium concentration remains below the contraction threshold and that the high fluorescence areas that we see must be highly compartmentalized, most probably in the nuclear region. As expected, high concentrations of ryanodine eliminated the initial rapid calcium increase associated with contraction, and interestingly, it also eliminated the first or slow-rapid cytosolic propagation as well. However the second slow calcium rise phase was preserved. This suggests that a ryanodine-sensitive calcium pool is involved in the mechanism of propagation of the slow-rapid Ca\(^2+\) wave through the cytosol. The apparently different intracellular distributions of receptors, RyR in the sarcoplasmic reticulum membranes and inositol 1,4,5-trisphosphate receptors (IP\(_3\)R), at least in some developmental stages, concentrated in membranes associated with the nuclei (7–9), points to the presence of two separate calcium release systems. We proposed that the role for the IP\(_3\)R could be to modulate cytosolic calcium concentrations within the appropriate levels, sub-cellular regions, and time scale required to activate nuclear calcium release. Slow calcium signals in these cells appear to be mediated by IP\(_3\) receptors and are likely to control phosphorylation cascades involved in regulation of gene expression. The aim of the present work was to determine the definitive role of RyRs, if any, in either component of the slow release process. To this aim, we compared the calcium signals in dyspedic muscle cells (1B5), which do not express any of the RyR isoforms and lack excitation-contraction (E-C) coupling (10–17) to C\(_2\)C\(_{12}\) cells, which have wild type calcium signals.

MATERIALS AND METHODS

Cell Cultures—Myoblasts of the immortalized dyspedic mouse myoblast cell line 1B5 (3) and the C\(_2\)C\(_{12}\) myoblast line (American Type Culture Collection, Manassas, VA) were cultivated in Dulbeco’s modified Eagle’s medium (1 g of glucose/liter), 10% heat-inactivated fetal calf serum, and 10% bovine serum (all Life Technologies, Inc.) in gelatin-covered dishes at 37 °C in 5% CO\(_2\). The serum was reduced to 2% horse serum after 2 days to induce cell maturation and fusion, and cells were studied 5–7 days after differentiation was initiated.

Intracellular Calcium—For intracellular calcium measurements at single-cell level, the myoblasts were cultured on glass coverslips to reach 80% confluence and then differentiated into myotubes by withdrawal of growth factors. Calcium images were obtained from myotubes
that were loaded with the fluorescence calcium dye fluo-3-acetoxymethylester (fluor-3AM; Molecular Probes, Eugene, OR) using an inverted confocal microscope (Carl Zeiss Axiolab 135 M-LSM Microsystems). Alternatively we observed calcium transients with an epifluorescence microscope (Olympus) equipped with a cooled CCD camera and image acquisition software (Scion Image program from NIH). Myotubes were washed three times with Krebs buffer (145 mM NaCl, 5 mM KCl, 2.6 CaCl$_2$, 1 mM MgCl$_2$, 10 mM Hepes-Na, 5.6 mM glucose, pH 7.4) to remove serum and loaded with 5.4 µM fluo-3 (coming from a stock in pluronic acid, 20% Me$_2$SO) for 30 min at room temperature. After loading, myotubes were washed for 10 min to allow the deesterification of the dye and used within 2 h. The coverslips were mounted in a 1-mL capacity plastic chamber and placed in the microscope for fluorescence measurements. After excitation with a 488-nm wavelength argon laser beam or filter, the fluorescence images were collected every 0.4–2.0 s and analyzed frame by frame with the data acquisition program of the equipment. A PlanAxio 60X (NA 1.4) objective lens was used. In most of the acquisitions, the image dimension was 512 × 120 pixels. Intracellular calcium was expressed as a percentage of fluorescence intensity relative to basal fluorescence (a value stable for at least 5 min in resting conditions). The increase in fluorescence intensity of fluo-3 is proportional to the ratio in intracellular calcium level (18). For experiments using inhibitors, U-72133 (Sigma), 2-aminoethylxydiphenyl borate (Aldemich), xestospongion C (Calbiochem), and ryanodine (Sigma) were used.

Digital Image Processing—Elimination of out-of-focus fluorescence was performed using the "auto-sharpen" deconvolution algorithm and Castleman’s (19) PSF (point spread function) theoretical model, as has been described previously (20). To quantify fluorescence, the summed pixel intensity was calculated on the section delimited by a contour. As a way of increasing efficiency of these data manipulations, action sequences were generated. To avoid the possible interference in the fluorescence by high potassium solution effects on the cellular volume, the area of fluorescent cell was determined by image analysis using adaptive contour and then creating a binary mask, which was compared with its bright-field image.

Binding of [H]ryanodine and [H]ryanodine—Radioiod binding assay for [H]ryanodine was determined as described (9). Briefly, confluent plates of C$_2$C$_12$ and dyspedic mouse cells lines 5–7 days after withdrawal of serum were washed three times with phosphate-buffered saline and homogenized with an ultrasonic homogenizer for 10–15 s. They were then incubated in a medium that contained 50 mM Tris-HCl, pH 8.4, 1 mM EDTA, 1 mM 2-mercaptoethanol, and different concentrations (10–200 nM) of [H]ryanodine (per-nyo-2-[H]ryanodine, specific activity 21.0 Ci/mmol, PerkinElmer Life Sciences, 800–1000 cpm/mmol) to 3 °C for 30 min. After incubation the reaction was stopped by centrifugation at 10,000 × g for 10 min (Heraus Biofuge 15R), the supernatant was aspirated, and the pellets were washed with phosphate-buffered saline and dissolved in NaOH (1 m) to measured the radioactivity. The non-specific binding was determined in the presence of 2 µM KCl (Sigma).

[1H]ryanodine binding was measured in C$_2$C$_12$ and dyspedic mouse cell homogenates as described (21). The incubation medium contained 0.5 mM KCl, 5 mM Hepes-Trio, pH 7.1, and 1 mM 54-methylimidodiphosphate or 5 mM adenosine trisphosphate. The samples were incubated with [1H]ryanodine (5–100 nM) for 90 min at 37 °C in the presence or absence of cold ryanodine (10 µM) for nonspecific binding.

Western Blots—Homogenate proteins were resolved in 7% SDS-polyacrylamide electrophoresis gels and transferred to nitrocellulose membranes for 2 h at 0.2 A. Primary antibody incubations and dilutions of 1:1000 of antibodies against either type 1 (Affinity Bioreagents) or type 3 (Transduction Laboratories) IP$_3$ receptor were carried out at 4 °C overnight. After incubation with horseradish peroxidase-conjugated secondary antibodies for 1.5 h, the membranes were developed by enhanced chemiluminescence according to the manufacturer’s instructions. After scanning the films, a densitometry analysis of the bands was performed with the Scion Image program from NIH.

Immunocytochemistry—Myotubes grown on coverslips were fixed in ice-cold methanol, blocked in phosphate-buffered saline containing 1% bovine serum albumin and 10% goat serum for 30 min and incubated with primary antibodies at 4 °C overnight. The primary antibodies obtained from commercial sources were raised against anti-ryanodine receptor monoclonal (Affinity Bioreagents) and anti-type 1 IP$_3$R (polyclonal Affinity Bioreagents). Anti-type-3 IP$_3$R antibody was raised against rabbits against a peptide corresponding to the carboxyl-terminal 16 amino acids of the rat type-3 IP$_3$R cDNA (CRRQRLGFVDVQNCMSR) (22). The antibody used in this study was affinity-purified using the immunogen peptide. The cells were then washed five times with phosphate-buffered saline/bovine serum albumin and incubated with the appropriate goat anti-mouse or goat anti-rabbit secondary antibody for 1 h at room temperature. After washing three more times, the coverslips were mounted in 90% glycerol, 0.1 mM Tris, pH 8.0, and 5 mg/mL p-phenylenediamine to retard photobleaching. The samples were evaluated in a scanning confocal microscope and phase contrast microscope and documented through computerized images. In most cases a nucleation staining was carried out with MTOX (Dr. C. Peters, Biocenter, University of Basel, Switzerland) or Rhodamine Red-X (Molecular Probes, Eugene, OR). Photobleaching was kept to a minimum.

Statistics—All data are expressed as mean ± S.D. Differences between basal and post-stimulated points were determined using a paired Student’s t test. p < 0.05 was considered statistically significant.

RESULTS

Intracellular Calcium Signals—Confocal microscopy was used to investigate fluo-3 fluorescence in both a RyR-expressing mouse muscle cell line (C$_2$C$_12$) and in dyspedic cells (1B5) in response to potassium depolarization. A typical effect of high potassium solution (47 mM K+) on intracellular calcium in C$_2$C$_12$ cells (Fig. 1A) consists of a fast (reaching the peak in less than 1 s) increase of fluo-3 fluorescence in the entire cell (n = 54 of 63); fluorescence slowly decreased during the next few seconds. After the first signal began to decline, a second, smaller signal was evident at about 10–20 s in 48% of the cells observed (n = 26). This signal usually propagates along a region of the cell, and fluorescence in some nuclei continues to increase after the wave passes them. The analysis of this particular cell allowed measurements of a given calcium signal in different regions of the myotube in order to study these phenomena (see below). The caffeine used is similar to the calcium transients described in cultured primary rat (5, 6) or mice (4) myotubes. Nuclear region signals appear to have a relative fluorescence change higher than the cytoplasmic signals, probably because fluo-3 is concentrated in the nucle (Polycomb, 6). Myotubes from the dyspedic (1B5) skeletal muscle cell line, which lacks ryanodine receptors, also displayed intracellular calcium increases upon K+ stimulation, but the kinetic pattern of this signal was different from that of their “normal” counterpart (Fig. 1B). As expected in dyspedic myotubes, the fast calcium signal was not present. However, a slow increase of calcium in the nuclei was apparent several seconds (mean time 8.2 ± 4.6 s range 2–18 s) after K+ stimulation. No propagation of this signal in the cytoplasm was evident. A total of 57 independent experiments using K+ were recorded in dyspedic myotubes; 43 of them (75%) demonstrated a significant slow
calcium rise in the nuclear region. It is interesting to note also a difference in duration of the slow calcium signal; the mean duration of Ca\(^{2+}\) increase in C\(_2\)C\(_{12}\) cells was 11.5 ± 6.0 s (range 2–26 s), whereas in dyspedic cells, the mean duration was significantly higher (27.8 ± 13.8 s range 8–42 s; p < 0.05).

In a more detailed study of the intracellular calcium increases produced by potassium stimulation in both cell lines, the relative changes in the fluorescence of a cell are displayed as a function of time. In Fig. 2A, the series of fluorescence images of a multinucleated C\(_2\)C\(_{12}\) myotube shown in Fig. 1A was analyzed; two different regions of equivalent areas of cytosol containing "responsive" or "unresponsive" nuclei were delimited. Within each contour, the summed intensity of all pixels in each image of the sequence was calculated (20). The fluorescence intensity of all pixels inside the pre-established contour was quantified for each of the images of the acquired series. When the time course of relative fluorescence for C\(_2\)C\(_{12}\) cells is analyzed (Fig. 2A), it is evident that the signal has at least two components. Fast fluorescence rise occurred simultaneously in both areas selected, indicating a very fast, propagated signal that spanned the whole cell in less than 1 s and slowly declined. When we analyze two sections separated by 33 μm, it can be seen that fluorescence rises in both areas at the same time (fast component). However, the slow component occurred as an oscillation, starting earlier in the left-side region (filled circles) and then propagated to the right (empty circles). As a distinct nucleus fluoresced in the right-hand region (see Fig. 2A, inset), it is possible to notice a shoulder in the curve, indicating the presence of both a faster (smaller) and a delayed component in this signal (empty circles). A similar analysis for dyspedic myotubes was also performed (Fig. 2B). 1B5 cells show a delayed, not propagated component of calcium rise that lasted longer than those of C\(_2\)C\(_{12}\) cells and was clearly higher in nuclear than in cytosolic regions (compare the intensity of the signal in two areas containing nuclei with that of a cytosolic area, Fig. 2B). In another subset of experiments, we tested a possible role of calcium influx on K\(^{+}\)-activated Ca\(^{2+}\) transients with confocal imaging. To minimize Ca\(^{2+}\) entry into myotubes, the experiments were performed in a low external Ca\(^{2+}\) solution containing 96 mM NaCl, 5 mM MgCl\(_2\), 2 mM KCl, 5 mM Heps, 0.5 mM EGTA. In seven independent experiments each of the calcium transients were clearly visible in both C\(_2\)C\(_{12}\) and 1B5 cells, and the spatial and temporal pattern of those signals were similar to those obtained in normal external calcium (data not shown).

Use of Inhibitors of the IP\(_3\) Pathway—To identify the calcium release systems involved in these signals, we measured calcium signals in both 1B5 and C\(_2\)C\(_{12}\) cells in the presence of known inhibitors of IP\(_3\)-mediated processes. In 1B5 cells (Fig. 3), the calcium rise was either completely blocked or greatly inhibited in the presence of 10 μM phospholipase C inhibitor U73122 (5 out of 6 experiments). The same happened for the slow part of the signal in C\(_2\)C\(_{12}\) cells (not shown). 100 μM xestospongin C, an IP\(_3\) receptor blocker (24), also almost completely inhibited the calcium rise in 6 out of 7 experiments with 1B5 cells. Finally, as was shown by Powell et al.\(^2\) in primary cultures, 50 μM cell-permeant modulator of the IP\(_3\)-signaling 2-aminoethoxydiphenyl borate (25) also inhibited the slow calcium sig-
nal in both cell lines (9 out of 12 experiments) but did not affect the fast calcium rise in C2C12 cells (not shown).

**C2C12 Incubated with Ryanodine—** When C2C12 cells were previously incubated with 20 μM ryanodine, the fast rise after potassium depolarization was completely abolished (Fig. 4), and a long-lasting increase of calcium fluorescence (mean duration 28 ± 6 s) was evident in the whole cell after a 6-s delay. Note that fluorescence intensity was particularly high at the level of the cell nuclei. It is also important to note in this case the complete lack of slow propagation of the signal, all nuclear fluorescence increasing at about the same time (Fig. 4).

**[3H]Ryanodine and [3H]IP3 Binding—** The presence of ryanodine receptor/calcium release channels (RyR channels) in C2C12 and 1B5 skeletal myotubes was studied using a radioligand assay. In C2C12 cells, [3H]ryanodine binding as a function of [3H]ryanodine concentration was hyperbolic (Fig. 5A). The Scatchard analysis for the specific binding component (inset, Fig. 5A) was fitted to a single family of receptors, with a high maximal binding capacity ($B_{max}$ = 0.88 pmol/mg of protein). [3H]Ryanodine binding to C2C12 myotubes is similar to that described for other skeletal muscle myotubes (6, 9, 26). On the other hand, under identical assay conditions, no specific [3H]ryanodine binding could be detected in dyspedic myotubes (1B5 cells), since no difference between total and nonspecific binding was found (Fig. 5B). This confirms the previously published results for this cell line (13). A second family of calcium release channels known to be targeted to the nucleus corresponds to inositol 1,4,5- trisphosphate receptors (27, 28). Expression of IP3Rs in C2C12 as well as 1B5 myotubes was determined by [3H]IP3 binding to myotube homogenates of C2C12 and 1B5 cells, respectively (Figs. 5, C and D). A saturating (specific) binding curve for [3H]IP3 was found in both cell lines. The Scatchard analysis for the specific binding component was fitted to a single family of receptors, with a high maximal binding capacity. Similar $K_d$ values were found in 1B5 and C2C12 myotubes, 61.8 ± 16.3 and 60.1 ± 22.2 nM, respectively, and the total amount of IP3 receptors ($B_{max}$) was the same in both cell types (3.12 versus 2.8 pmol/mg of protein, respectively).

**Western Blot Analysis—** The presence of different IP3 isoforms was investigated in C2C12 and 1B5 cell myotubes by
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**Immunoblotting**—Polyacrylamide gel electrophoresis-separated total cell lysates (Fig. 6). Cerebellum proteins (first lane, left panel) and HeLa cells (third lane, right panel) were used as positive controls for type 1 and type 3 IP₃ receptors, respectively. Both C₂C₁₂ and 1B5 cell myotubes co-expressed both type 1 and type 3 IP₃ receptor isoforms (Fig. 6) in nearly equal proportions. However, a densitometry analysis showed that 1B5 myotubes expressed higher levels of both proteins, which were estimated as a fraction of total protein compared with C₂C₁₂ myotubes (Fig. 6, lower panels).

**Immunocytochemistry**—The intracellular localization of IP₃ receptor isoforms was monitored by immunofluorescence labeling and confocal microscopy. As shown in Fig. 7, A and B, fluorescence due to type 1 IP₃R presents a similar pattern in both C₂C₁₂ and 1B5 myotubes. Immunoreactivity is seen primarily in the nuclear envelope and some internal nuclear structures. However, the internal nuclear labeling is most likely to be nonspecific because these structures are seen when the cells are incubated with preimmune serum in place of the primary antibody (6). The staining of type 1 IP₃R appears to be continuous around the nuclear envelope. This was confirmed by sectioning the cells in z axis using scanning confocal microscopy (data not shown). On the other hand, the antibody against type 3 IP₃R shows a significant amount of internal nuclear labeling (Fig. 7, C and D) especially in 1B5 myotubes, where it is expressed at high levels. There is also a punctate pattern of type 3 staining throughout the cellular matrix in both cell types. C₂C₁₂ myotubes showed specific immunoreactivity after exposure to anti-RyR antibodies (Fig. 7E) and demonstrated that RyR, when expressed, did not co-localize with IP₃ receptor. This indicates that there is a different spatial distribution of these proteins in these myotubes. As

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**Fig. 5.** [3H]IP₃ and [3H]ryanodine binding in C₂C₁₂ and 1B5 cells. A, the microsomal membrane fraction of C₂C₁₂ cells shows specific [3H]ryanodine binding (nonspecific binding, not shown, was subtracted to all points); data was fitted to a Kᵦ of 17.7 ± 2.9 nM and a maximal binding capacity of 0.88 pmol/mg of protein. Nonspecific binding was measured in the presence of 10 μM ryanodine. B, [3H]ryanodine binding to 1B5 cell homogenates. The specific ryanodine binding component is absent. No differences were found between total and nonspecific curves. C, a microsomal membrane fraction of C₂C₁₂ myotubes binds [3H]IP₃ with a Kᵦ of 60.1 ± 22.2 nM and a maximal binding capacity of 2.80 pmol/mg of protein, the fit to the equilibrium binding curve implies a single type of receptor. D, [3H]IP₃ binding to 1B5 cell homogenates. The Kᵦ (61.8 ± 16.3 nM) and Bₘₐₓ (3.12 pmol/mg of protein) were determined by best fitting of data. The nonspecific binding were measured in the presence of 2 μM IP₃. The saturation isotherms were fitted to a single class of binding sites.

**Fig. 6.** SDS-polyacrylamide gel analysis of type 1 and type 3 IP₃ receptor. A representative record of at least three independent preparations is presented. Both type 1 (left panel) and type 3 (right panel) IP₃ receptor were visualized by Western blot analysis in total cell lysates of C₂C₁₂ (first lane) and 1B5 (second lane) myotubes. Cerebellum proteins (first lane, left panel) and HeLa cells (lane 3, right) were used as standard for type 1 and type 3, respectively. Aliquots of these homogenates (10 μg) were analyzed on 7% SDS-polyacrylamide gel, and later the proteins were detected using commercial antibodies. Type 3 IP₃ immunoreactivity was higher in 1B5 cells as compared with total lysates obtained from C₂C₁₂. MW, molecular mass.

**Fig. 7.** Immunocytochemistry. Fluorescence immuno-labeling of types 1 and 3 IP₃ receptors in C₂C₁₂ myotubes. A and C, epitope affinity-purified rabbit polyclonal antibody to IP₃R type 1 labels the nuclear envelope region and some internal nuclear structures; the latter label is nonspecific since preimmune serum labels only internal nuclear structures (6). On the other hand, antibody to type 3 IP₃R shows internal nuclear labeling (C). Myotubes show very faint fluorescence when incubated with the fluorescence-conjugated secondary antibody alone (not shown) or when incubated in the presence of the antigenic peptide (F; no nuclear label was detected in this case (arrows)). Anti-ryanodine receptor, together with anti-type 1 IP₃R staining is shown in C₂C₁₂ (E). A monoclonal antibody against ryanodine receptor was used. Type 1 and 3 IP₃ receptors in 1B5 myotubes with fluorescence immuno-labeling were also evident. B and D, the spatial distribution of these receptors was not different compared with control C₂C₁₂ cells, but label intensity was usually higher. Calibration bar for all images is 15 μm.
**Calcium Signal in Normal and Dyspedic Myotubes**

Our data show that in the absence of RyR expression, cultured dyspedic skeletal myotubes retain the slow delayed intracellular calcium transient that is seen as a second phase of release after a K⁺ depolarization in normal myotubes expressing RyR. This slow delayed release appears to be due to the presence of IP₃ receptors that are expressed in both types of cells. Our experimental evidence to support this hypothesis include the following: 1) Myotubes from a normal muscle cell line like C₂C₁₂ show fast and slow calcium signals that follow high potassium depolarization, as those described for myotubes in primary culture. 2) B₅, dyspedic muscle cells, which do not express any of the ryanodine receptor isoforms (confirmed by [³H]ryanodine binding and immunocytochemistry), show a calcium increase induced by K⁺ depolarization that seems to be especially important at the level of the nucleus. The kinetics of this transient is compatible with the slow signal present in rat and mouse primary cultures (6, 9, 20) or C₂C₁₂ cells. Both the fast calcium transient, which is responsible for excitation-contraction coupling, and the fast-slow wave, which is associated with signal propagation, are absent in B₅ myotubes. The fast calcium signal is restored in these cells when RyR1, but not RyR3, is expressed (11, 13, 16). 3) Both [³H]IP₃ binding and Western blot analysis show the presence of IP₃ receptors in both B₅ and C₂C₁₂ muscle cells. The presence of IP₃,R isoforms was confirmed by immunocytochemistry. Type 1 IP₃,Rs were localized preferentially in the nuclear envelope, and both type 1 and type 3 IP₃,R immunoreactivity was higher in B₅ cells compared with C₂C₁₂. 4) In both B₅ and C₂C₁₂ cells, K⁺ depolarization resulted in an increase in IP₃ mass. The kinetics of the IP₃ mass transient is compatible with IP₃ release being the precursor to the calcium transient.

_Binding studies confirm that IP₃,Rs are more abundant than RyRs in cultured muscle cells, as has been previously reported (6, 9, 26). It would be interesting to know the relative amounts of the sub-types of IP₃,Rs present in these cells. The higher staining of type 3 antibodies in Western blots is not a direct proof of higher antigen concentration, since the antibodies were directed against different epitopes and their avidity for the antigen is unknown. The binding studies likewise, do not directly address the matter. Values of Kᵣ for the different isoforms reported in the literature are around 1 nM for type 1 (or 2) and about 40 nM for type 3 (29). The value we found by fitting our data to a single receptor type curve (our Scatchard plots, with a limited number of points, do not show a clear second component) is around 60 nM for both cell lines. So, if Kᵣ values for IP₃,Rs in muscle cells are comparable with those described for receptors purified from other cells, we may have a high proportion of type 3 IP₃,R as compared with type 1 in our cells. Such a high type 3 receptor content could mask a small proportion of high affinity components in the Scatchard plot._

We have previously shown that K⁺ depolarization produces a pattern of calcium signals on cultured skeletal muscle myotubes, characterized by a fast and a slow intracellular calcium increase (5, 6). We postulated that the fast response is dependent on ryanodine receptors, whereas the second, slow response...
appears associated to cell nuclei and mediated by IP₃. The results of the present study support this hypothesis and demonstrate a functional role for IP₃ as a modulator of calcium-mediated cellular processes in the skeletal muscle cell, occurring on the time scale of seconds. The fact that the slow calcium signal can be inhibited by various substances known to interfere with either IP₃ generation or IP₃ action (24, 25) and the fact that these compounds do not interfere with the fast signals in C₆C₁₂ cells provide further support in this direction. The lack of a fast calcium transient and the presence of a delayed, slow calcium transient seen in 1B5 myotubes is similar to what was seen in C₆C₁₂ myotubes and (previously) in primary rat C₂C₁₂ cells. This finding is intended to regulate local Ca²⁺ concentration within the cell. Whatever the mechanism, the existence of this signal certainly suggests the presence of different calcium release compartments in muscle cells, the nuclear envelope being one of them (7, 32, 34). The precise role of the subtypes of IP₃ and ryanodine receptors in what appears to be a complex time and space signaling pattern remains to be established.

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