Nuclear inositol 1,4,5-trisphosphate receptors regulate local Ca^{2+} transients and modulate cAMP response element binding protein phosphorylation

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Journal of Cell Science 118, 3131-3140

Summary

Several lines of evidence indicate that increases in nuclear Ca^{2+} have specific biological effects that differ from those of cytosolic Ca^{2+}, suggesting that they occur independently. The mechanisms involved in controlling nuclear Ca^{2+} signaling are both controversial and still poorly understood. Using hypotonic shock combined with mechanical disruption, we obtained and characterized a fraction of purified nuclei from cultured rat skeletal myotubes. Both immunoblot studies and radiolabeled inositol 1,4,5-trisphosphate [IP_3] binding revealed an important concentration of IP_3 receptors in the nuclear fraction. Immunofluorescence and immunoelectron microscopy studies localized type-1 and type-3 IP_3 receptors in the nucleus with type-1 receptors preferentially localized in the inner nuclear membrane. Type-2 IP_3 receptor was confined to the sarcoplasmic reticulum. Isolated nuclei responded to IP_3 with rapid and transient Ca^{2+} concentration elevations, which were inhibited by known blockers of IP_3 signals. Similar results were obtained with isolated nuclei from the 1B5 cell line, which does not express ryanodine receptors but releases nuclear Ca^{2+} in an IP_3-dependent manner. Nuclear Ca^{2+} increases triggered by IP_3 evoked phosphorylation of cAMP response element binding protein with kinetics compatible with sequential activation. These results support the idea that Ca^{2+} signals, mediated by nuclear IP_3 receptors in muscle cells, are part of a distinct Ca^{2+} release component that originates in the nucleus and probably participates in gene regulation mediated by cAMP response element binding protein.

Key words: Skeletal muscle, Myonuclei, Inositol 1,4,5-trisphosphate receptors, Nuclear envelope, Transcription factors, Gene expression

Introduction

Ionized calcium is a versatile second messenger that mediates a wide variety of biological processes in cells, such as secretion, contraction, differentiation, proliferation, programmed cell death and gene expression (reviewed by Brini and Carafoli, 2000; Berridge et al., 2003). Ca^{2+} concentration changes in the cytoplasm and nucleus are often assumed to occur more or less simultaneously owing to the lack of diffusion barriers between them (reviewed by Bootman et al., 2000). However, several studies indicate that despite the high permeability of nuclear pores, an active Ca^{2+} gradient between the nucleus and cytoplasm is present (Al-Mohanna et al., 1994; Badminton et al., 1996; Leite et al., 2003). Moreover, several lines of evidence suggest that increases in nuclear Ca^{2+} have specific biological effects that differ from those of cytosolic Ca^{2+} (Hardingham et al., 1997; Quesada et al., 2002). The mechanisms involved in controlling nuclear Ca^{2+} signaling are both controversial and still poorly understood. The nucleus is surrounded by a double membrane, the nuclear envelope, which is structurally and functionally related to the endoplasmic reticulum (ER) (Lanini et al., 1992). Thus, it is capable of accumulating Ca^{2+} inside its lumen (Gerasimenko et al., 1995). In addition, it shares certain characteristics with the ER, for example, the presence of a Ca^{2+}-Mg^{2+}-ATPase in the external nuclear envelope (Kaprielian and Fambrough, 1987), and Ca^{2+} binding proteins such as calreticulin (Camacho and Lechleiter, 1995), calbidin-D_{28k} and nucleolin (Malviya et al., 1990). All these properties enable the nucleus to store Ca^{2+}. Also common to both organelles are IP_3 receptors (IP_3Rs) and ryanodine receptors (RyRs) presumably responsible for Ca^{2+} release in the nucleoplasm.

IP_3Rs are a family of Ca^{2+}-permeable channels composed of three isoforms that differ in amino acid sequence, affinity for IP_3 and modulation by Ca^{2+} (Joseph, 1996). The subcellular distribution of IP_3R isoforms is also different in many cells, and the distribution of nuclear isoforms in particular remains unclear. The IP_3Rs in the nucleus could be activated locally after generation of IP_3 by the nuclear phospholipase C (PLC),

which hydrolyzes phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2] present in the nuclear membrane (Irvine, 2000). Moreover, the nucleus has other components needed for IP_3 metabolism, as PtdIns 3-kinases, PtdIns 4-kinases, PtdIns(4)*P* 5-kinases and diacylglycerol kinases (Maraldi et al., 1999). Thus, the nucleus has the capability to regulate nucleoplasmic Ca^{2+}, independently of cytoplasmic Ca^{2+} levels.

Nuclear Ca^{2+} increases have been suggested to control the cAMP response element binding protein (CREB)-mediated gene expression in neurons (Hardingham et al., 1997; Hardingham et al., 2001). However neither the source of Ca^{2+}, nor the participation of nuclear channels is clear. It was recently reported that nuclear K^+-ATP-dependent channels triggered by nuclear Ca^{2+} increases induced CREB phosphorylation in isolated nuclei (Quesada et al., 2002). However, the possible participation of IP_3Rs in modulating the CREB phosphorylation response in nuclei is unknown.

Our previous findings of Ca^{2+} signals mediated by IP_3 with an important nuclear component, following depolarization of skeletal myotubes (Jaimovich et al., 2000; Powell et al., 2001; Eltit et al., 2004), or of different muscle cell lines (Estrada et al., 2001) led us to consider the possibility that IP_3Rs have a role in the generation of nuclear Ca^{2+} signals. It has been suggested that the IP_3-mediated Ca^{2+} increase regulates early gene expression (Powell et al., 2001; Araya et al., 2003; Carrasco et al., 2003).

Materials and Methods

Reagents

Antibodies directed against the whole molecule or epitope-purified IP_3R type-1, calsequestrin and triadin were obtained from Affinity Bioreagents (Golden, CO). Antibodies against IP_3R type-2, total CREB, FITC-conjugated goat anti-rabbit IgGs and TRITC-conjugated goat anti-mouse IgG were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against rat type-3 IP_3R were previously described (Ramos-Franco et al., 1998). Antibodies against phosphorylated forms of CREB were from Cell Signaling Technology (Beverly, MA). Secondary HRP-conjugated anti-rabbit antibodies were from Pierce Biotechnology (Rockford, IL). Antibodies against LAP2 were purchased from Transduction Laboratories. Enhanced chemiluminescence (ECL) reagents were from Pierce Biotechnology or Amersham Biosciences (Piscataway, NJ) and 2-aminoethoxydiphenyl borate (2-APB) was from Sigma (St Louis, MO).

Cell culture and immunostaining

Muscle cells in primary culture and myoblasts of the immortalized dyspedic mouse cell line 1B5 (kindly provided by Paul Allen, Brigham and Women Hospital, Boston, MA) were prepared as described previously (Jaimovich et al., 2000; Moore et al., 1998; Takeshima et al., 1994). Immunocytochemistry of isolated nuclei and cultured cells was performed essentially as previously reported (Powell et al., 1996). Briefly, cells and nuclear monolayers placed on coverslips were fixed with methanol at –20°C for 12 minutes, blocked in 1% BSA and incubated with primary antibodies overnight at 4°C. Cells or isolated nuclei were then washed and incubated with secondary antibody for 1 hour at room temperature. Coverslips were mounted in Vectashield (Vector Laboratories) for confocal microscopy and representative images were acquired (Carl Zeiss Axiovert 135, LSM Microsystems). Negative controls had only secondary antibodies applied. For type-3 IP_3R, the pre-immune serum was also used. The images reproduced were manipulated in Adobe Photoshop™ to improve clarity; no information was added or deleted by those adjustments.

Isolation of nuclei

Highly purified nuclei were obtained by the combined use of a hypotonic shock and mechanical disruption in a Dounce homogenizer as previously reported (Martelli et al., 1992). Briefly, cells (10×10^6) were washed in saline phosphate buffer and incubated in 0.1 % trypsin (v/v) for 20 minutes at 37°C and later scraped off using a rubber policeman. Cells were precipitated at 4000 ***g*** to eliminate trypsin. The resulting cell pellet was suspended in hypotonic buffer (10 mM Tris-HCl, pH 7.8, 10 mM β-mercaptoethanol, 0.5 mM PMSF, 1 μg/ml aprotinin, leupeptin and pepstatin). After 20 minutes on ice the swollen cells were broken in a Dounce homogenizer. The nuclear pellet was obtained by centrifugation at 500 ***g*** for 6 minutes at 4°C and was then washed in 10 mM Tris-HCl, pH 7.2, 2 mM $MgCl_2$ plus the protease inhibitors. Finally the nuclear pellet was resuspended in 10 mM HEPES-Tris-HCl (pH 7.6), 110 mM KCl, 1 mM $MgCl_2$ and protease inhibitors. Nuclear integrity was checked by electron and confocal microscopy. After the nuclear pellet was obtained, a 'crude microsomal fraction' was obtained as described (Jaimovich et al., 2000) and this fraction was used for the [^{3}H]IP_3 binding experiments.

Immunoelectron microscopy

Purified myonuclei were centrifuged for 10 minutes at 4000 ***g*** and the nuclear pellet was immediately fixed for 1 hour at room temperature with 4% formaldehyde and 0.1% glutaraldehyde (Polyscience) in phosphate buffer, pH 7.4. The nuclear pellet was dehydrated in a graded ethanol series and embedded in LR-White resin (Electron Microscopy Sciences, Hatfield, PA). All immunolabeling was carried out at room temperature. Silver-grey thin sections were treated with 0.1% sodium borohydride (Sigma Chemical, St Louis, MO) PBS solution for 15 minutes. Non-specific labeling was blocked with PBS solution containing 5% acetylated BSA, 5% normal goat serum and 0.1% coldwater fish skin gelatin (Sigma). Samples were incubated with the same polyclonal rabbit anti-type-1 or type-3 IP_3R antibodies described above. Grids were then incubated for 2 hours with goat anti-rabbit IgGs conjugated to 10 nm colloidal gold particles (BioCell, Cardiff, UK). After thorough washing, samples were fixed with 2.5% glutaraldehyde (Polyscience) in PBS and post-fixed with 1% osmium tetroxide vapors. Finally, grids were counterstained with 2.5% uranyl acetate and 1% lead citrate and examined using a Philips CM-10 electron microscope.

[^{3}H]IP_3 binding

Radioligand binding assays for [^{3}H]IP_3 were performed as previously described (Liberona et al., 1998). Briefly, isolated nuclei from confluent plates of dyspedic mouse cell lines, 5-7 days after withdrawal of serum, were washed three times with PBS and homogenized with a Dounce homogenizer. They were then incubated in a medium that contained 50 mM Tris-HCl, pH 8.4, 1 mM EDTA, 1 mM 2-mercaptoethanol, and 10-200 nM [^{3}H]IP_3 (D-[2-^{3}H]-*myo*-inositol 1,4,5-trisphosphate), specific activity 21.0 Ci/mmol (DuPont, NEN), 800-1000 cpm/pmol at 4°C for 30 minutes. After incubation, the reaction was stopped by centrifugation at 10,000 ***g*** for 10 minutes, the supernatant was aspired and the pellets were washed with PBS and dissolved in 1M NaOH to measure radioactivity. Nonspecific binding was determined in the presence of 2 μM IP_3 (Sigma).

Western blot analysis

Cells were lyzed in 60 μl ice-cold lysis buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 5 mM Na_3VO_4, 20 mM NaF, 10 mM NaPP and a protease inhibitor cocktail

(Calbiochem). Cell lysates were sonicated, incubated on ice for 20 minutes and centrifuged to remove debris. Protein concentration of the supernatants was determined with BSA as standard. Lysate proteins were suspended in Laemmli buffer, separated in 10% SDS-polyacrylamide gels and transferred to PDVF membranes (Millipore). Blocking was at room temperature for 1 hour in 3% fat-free milk, and the membranes were incubated overnight with the appropriate primary antibody. Immunoreactive proteins were detected using ECL reagents (Pierce Biotechnology, Rockford, IL) according to the manufacturer's instructions. For CREB analysis, the films were scanned, and densitometry analysis of the bands was performed using the Scion Image program (http://www.scioncorp.com). To correct for loading, membranes were stripped in buffer containing 62.5 mM Tris-HCl, pH 6.8, 2% SDS and 50 mM β-mercaptoethanol at 50°C for 30 minutes, and reprobed with the corresponding antibodies.

Confocal microscopy and fluorimetry for Ca^{2+}

Nuclei were pre-incubated in a resting solution containing 125 mM KCl, 2 mM K_2HPO_4, 50 mM HEPES, 4 mM $MgCl_2$ and 0.1 mM EGTA (with <5 nM free Ca^{2+}), plus 5.4 μM fluo-3/AM (Molecular Probes, Eugene, OR) for 45 minutes at 4°C and then were incubated in a solution containing 140 mM KCl, 10 mM HEPES, 1 mM $MgCl_2$, 100 μM EGTA, 75 μM $CaCl_2$ with 200 nM free Ca^{2+} (estimated using the Winmaxc program, http://www.stanford.edu/~cpatton/winmaxc2.html) for loading. Loaded nuclei were washed and centrifuged, mounted in a 1 ml capacity perfusion chamber and placed in the microscope stage of a confocal laser-scanning system (Carl Zeiss Axiovert 135 M, LSM Microsystems) for fluorescence measurements. Nuclei were then stimulated with 10 μM IP_3 and fluorescent images were collected every second and analyzed frame by frame with a data acquisition program. The same protocol was used when the nuclei were stimulated in the presence of 50 μM 2-APB an inhibitor of IP_3 signals. Fluorimetric experiments were also performed. A suspension of nuclei was loaded with either mag-fluo-4 or fluo-4 dextran under the same conditions described for fluo-3/AM above. Fluorescence was measured using a multi-label reader Mithras LB 940 (Berthold technologies, Bad Wildbad, Germany).

Data analysis

All experiments were performed a minimum of three times. Results are expressed as mean±s.e.m. The significance of any differences among treatments was evaluated using the Student's *t*-test for paired data, or analysis of variance followed by Dunnett's post-test for multiple comparisons.

Results

Expression and distribution of three types of IP_3R in skeletal myotubes

To determine which types of IP_3R are expressed in primary rat skeletal myotubes, western blot analysis was performed using selective antibodies against type-1, type-2 and type-3 IP_3R. All three IP_3Rs are expressed with a relative molecular mass of ~260 kDa (Fig. 1A). No crossreactivity of the antibodies with proteins other than IP_3Rs was seen. In each case the same amount of protein was loaded. The distribution of receptor types was then investigated by immunocytochemistry. Type-1 IP_3R complete antibody labels young myotubes very strongly with either a dotted, or a continuous pattern in the nuclear envelope region (Fig. 1B and Fig. 2A). The same labeling pattern was seen when an epitope affinity-purified antibody was used (not shown), confirming the previously reported distribution (Jaimovich et al., 2000). In addition, the striated

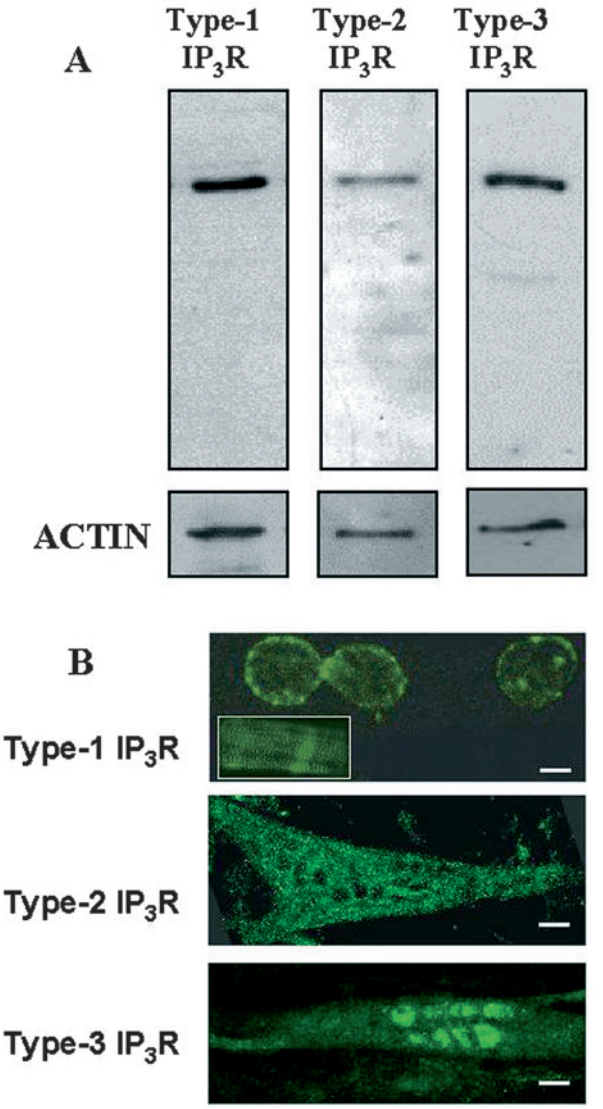

Fig. 1. Expression and distribution of IP_3 receptor isoforms in skeletal myotubes. (A) Representative western blots of six different experiments showing all three types of IP_3Rs (IP_3R1, IP_3R2 and IP_3R3) expressed in myotubes with a molecular mass of ~260 kDa. No crossreactivity was seen. To correct for loading the blots were probed with an antibody that recognizes actin. (B) Upper panel, type-1 IP_3R labeling is distributed in a dotted pattern in the nuclear envelope region. Inset, labelling of a differentiated myotube; striations correspond to limited regions of sarcoplasmic reticulum. Center panel, labeling of type-2 IP_3R is distributed throughout the reticulum and absent from nuclei. Lower panel, labeling of type-3 IP_3R is concentrated in nuclei, mainly in condensed structures and also occurs faintly in the cytoplasm. Bars, 5 μm (top), 25 μm (center, bottom).

pattern corresponding to longitudinal sarcoplasmic reticulum immunostaining (Powell et al., 2001) was observed (Fig. 1 inset and Fig. 2A). Type-2 IP_3R label is widely distributed in the reticulum throughout the cell with a regular reticular pattern (Fig. 1B, see also Fig. 2B). Staining was absent in the nuclei (observed as dark spaces in the center of myotubes in Fig. 1B). In order to determine the presence of type-3 IP_3R, two selective antibodies raised against specific epitope-regions

in both the C-terminus and N-terminus (Ramos-Franco et al., 1998) were used. Both antibodies specifically labeled structures both around and within the nucleus (Fig. 1B and Fig. 2C), whereas the cytoplasm showed much fainter labeling. In order to confirm the specificity of these antibodies, cells were incubated with pre-immune serum and showed no staining in the nuclear region (data not shown).

Type-1 IP_3R colocalizes with a specific marker of the inner nuclear membrane

To examine whether IP_3R localization in the nucleoplasm corresponds to invaginations of the inner nuclear membrane, as described for the nucleus of a glioma cell line (Lui et al., 1997), or are part of an independent structure, double immunostaining of IP_3R was performed with LAP2, a specific inner nuclear membrane protein marker. LAP2 showed a continuous staining pattern around the nuclei (Fig. 2), and also in invaginations of the inner nuclear membrane towards the nucleoplasm. Superimposition of type-1 IP_3R with LAP2 labels showed a high colocalization, suggesting that type-1 IP_3R is located in the nuclear envelope (Fig. 2A, lower panel).

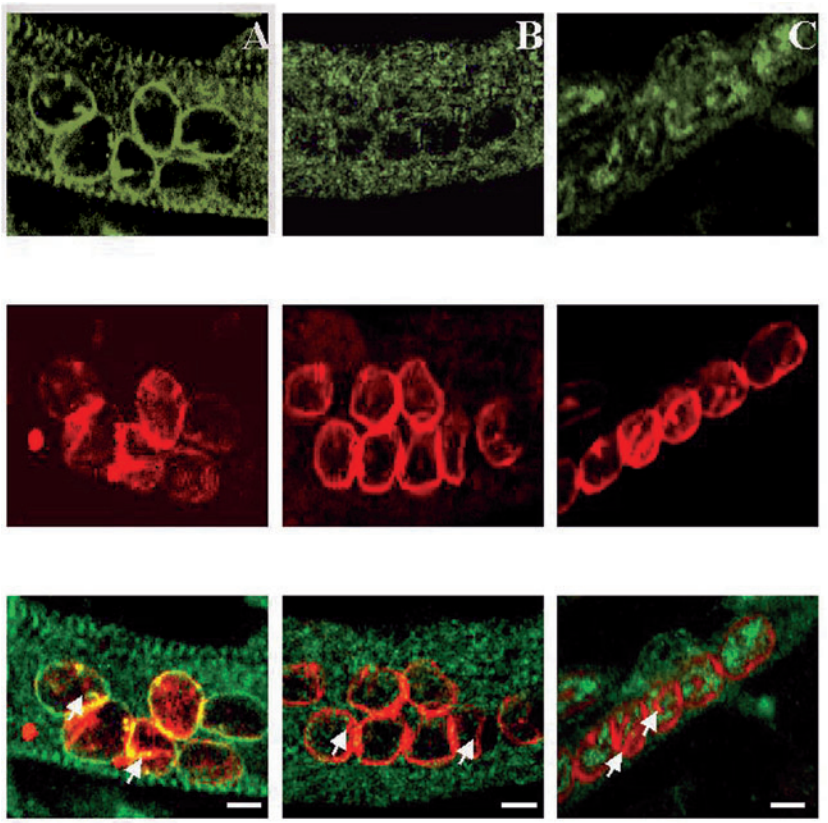

Fig. 2. Distribution of IP_3 receptor isoforms in myonuclei. Co-immunofluorescence analysis of IP_3 receptor isoforms with LAP 2 (a specific marker for the inner nuclear membrane). (A) Labels for both type-1 IP_3R (upper image), and LAP 2 (center image) in the nuclear envelope were superimposed (yellow in bottom panel). (B) The labels for both type-2 IP_3R (upper image) and LAP 2 (middle image) in the reticulum and nuclei respectively did not colocalize when images were superimposed (bottom image). (C) Immunolabel for type-3 IP_3R (upper image) and LAP2 (middle image) show no colocalization (bottom image). Arrows show invaginations of the nuclear envelope towards the nucleoplasm colocalizing with type-1 IP_3R, but not with either type-2 or type-3 IP_3R. Bar, 10 μm.

As expected, type-2 IP_3R did not colocalize with LAP2, confirming that type-2 IP_3R is confined to the reticulum (Fig. 2B, lower panel). Type-3 IP_3R generally showed no colocalization with LAP2 in the nuclear envelope and invaginations, but immunostained the nucleoplasm with a reticular pattern in areas not labeled with LAP2 (Fig. 2C, lower panel). However, in a few cells (not shown), partial colocalization with LAP2 was detected. This suggests that type-3 IP_3R labels some unknown nucleoplasmic structure that may correspond to the recently described nucleoplasmic reticulum (Echevarria et al., 2003).

Characterization of isolated myonuclei

To further substantiate the observation that IP_3Rs are present in the nucleus, we characterized a preparation of purified nuclei of cultured cells with minimal contamination from other organelles whilst conserving nuclear integrity. The isolation of nuclei was based on the application of a hypotonic shock combined with mechanical disruption (Martelli et al., 1992). The specific inner nuclear membrane marker LAP2 shows integrity and homogeneity of the preparation (Fig. 3A). In addition, immunoblot analyses were performed to determine the purity of the nuclear fractions. Two ER markers, triadin and calsequestrin were tested; these proteins were absent in the nuclear fraction. Furthermore, an important amount of the inner nuclear membrane protein LAP2, was evident in the nuclear fraction, whereas its presence in the cytosolic fraction was minimal (Fig. 3B). In all cases, the same amount of protein was loaded. Finally, integrity and purity of the nuclear fractions were examined by electron microscopy. The nuclear membrane integrity and the absence of detritus were evident in isolated myonuclei (Fig. 3C). The continuity of the nuclear membrane was only disrupted by the presence of nuclear pores (Fig. 3D). Other organelles like mitochondria or ER vesicles were absent from the preparation (data not shown).

Nuclear expression and localization of IP_3R isoforms

We measured [^{3}H]IP_3 binding to nuclear and cytosolic fractions and found saturating (specific) binding curves in both cases. Interestingly, more IP_3Rs appear to be located in the nuclear fraction compared to the cytosolic fraction. The Scatchard analysis for the nuclear fraction (Fig. 4A, upper panel) was fitted to a single receptor site, with a K_d of 82.55±20.29 nM and the total amount of IP_3Rs (B_{max}) was 2.41 pmol/mg protein. On the other hand the cytosolic fraction also shows a similar K_d, (Fig. 4A, lower panel), but with a smaller total amount of IP_3Rs (1.64 pmol/mg protein).

We also examined whether IP_3R isoforms conserved their expression and distribution in a fraction of isolated myonuclei. Immunoblotting of the nuclear and cytosolic fraction shows that

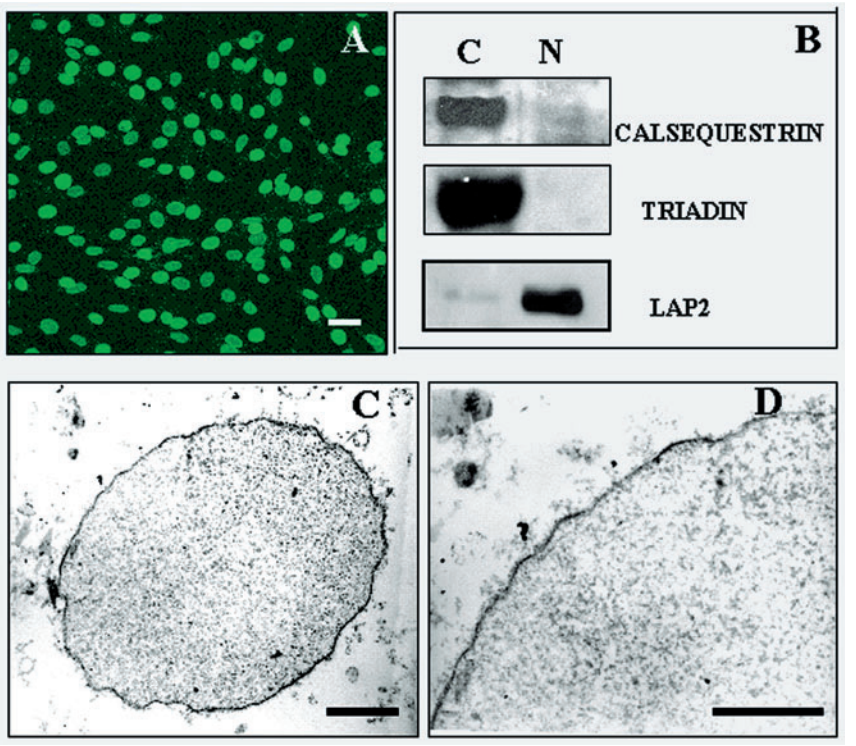

Fig. 3. Characterization of an isolated nuclear fraction from skeletal myotubes. (A) Fluorescence images of isolated myonuclei from a primary culture immunolabeled with LAP2 showing the integrity of the nuclei after the purification procedure. (B) Representative western blots of three different experiments showing minimal contamination of the nuclear fraction with the sarcoplasmic reticulum markers calsequestrin (top), and triadin (middle), but enriched with the nuclear marker LAP2 (bottom). (C) Transmission electron micrograph of a purified nucleus, showing the conserved nuclear structure. (D) High magnification electron micrograph showing the continuity and integrity of the nuclear membrane. Bar, 20 μm (A); 2 μm (C); 0.5 μm (D).

type-1 IP_3R was present in both fractions and clearly enriched in the nuclear fraction (Fig. 4B). Similarly, the type-3 isoform was always present and enriched in the nuclear fraction, but was also detected in the cytoplasmic fraction (Fig. 4B). Type-2 IP_3R was present in the cytoplasmic fraction and completely absent from the nuclear fraction (Fig. 4B). We then determined whether IP_3R isoforms located and distributed in isolated nuclei in a similar way to that shown for the whole cell. Using the same antibodies described above, IP_3R isoforms were detected by immunocytochemistry and we studied their localization in isolated nuclei. Nuclei were stained with type-1 IP_3R antibodies, revealing their main localization within the nuclear envelope with either a solid or more speckled distribution (Fig. 4C). Type-3 IP_3R was distributed in the nucleoplasm, with a patchy pattern (Fig. 4D), whereas type-2 IP_3R was not detected in isolated nuclei (not shown). Although previous observations in other cells (Leite et al., 2003; Adebanjo et al., 2000) have shown the presence of IP_3Rs in the nucleus, the isoform distribution appears to be different to that described here.

We also looked in the nuclear fraction of primary myotubes for the expression of RyRs, a well-known intracellular Ca^{2+} channel in skeletal muscle cells. Immunoblot and immunocytochemistry analyses revealed that RyRs are present in the nuclear fraction and are located in the nuclear envelope (data not shown).

Immunogold cytochemistry was carried out to determine the fine nuclear localization of IP_3R isoforms. Type-1 IP_3R

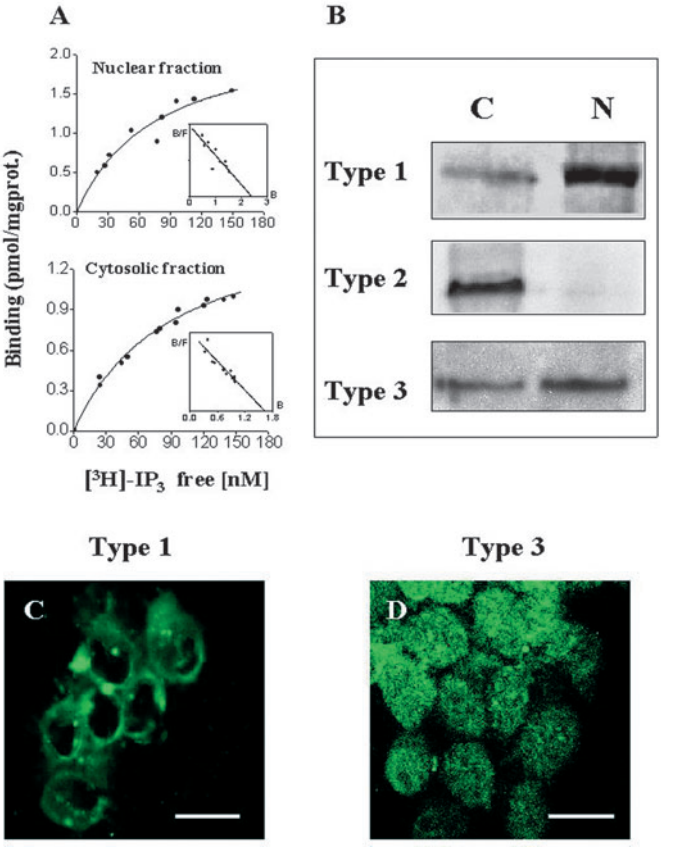

Fig. 4. Expression of IP_3 receptor isoforms in nuclear (N) and cytosolic (C) fractions of myotubes and their distribution in isolated nuclei. (A) Nuclear and cytosolic fractions of cultured myotubes bind [^{3}H]IP_3 with a K_d 82.55±20.29 nM and 91.2±11.4 nM, respectively, and have a maximal binding capacity of 2.41 and 1.64±0.10 pmol/mg protein [see Scatchard plots (insets)]. The non-specific binding was measured in the presence of 2 μM cold IP_3. (B) Representative SDS-polyacrylamide gel analysis (from three independent preparations) of nuclear and cytosolic fractions. Type-1 IP_3R was enriched in the nuclear fraction, but was also present in the cytosolic fraction (upper panel); type-2 IP_3R was present only in the cytosolic fraction (middle panel), and type-3 IP_3R was enriched in the nuclear fraction, but was also present in the cytosolic fraction (bottom panel). In all cases the same concentration of protein was loaded. (C,D) Immunofluorescence of isolated myonuclei revealed that type-1 IP_3 receptor conserved the localization in the nuclear envelope (C) and that type-3 IP_3R was distributed with a speckled pattern in the nucleoplasm (D). Bar, 15 μm.

reacting gold particles were found mainly in clusters of varying numbers (from 2 to 30 particles) (Fig. 5A). More than half of the gold particles found were in clusters of three or more particles, 72 % of particles being in the vicinity of one or more particles. They were preferentially located near the inner nuclear membrane with some associated with structures in the nucleoplasm. Surprisingly, a considerable number of particles appeared to be associated with the nucleolus. It was also possible to see a few particles associated with the outer nuclear membrane (not shown). When the number of particles in the different nuclear compartments was quantified, we found that approximately 47% were associated with the nucleoplasm, 21% to the nucleolus, 25% to the inner nuclear membrane and 7% to the outer nuclear membrane. Gold particles reacting with type-3 IP_3Rs were also localized near the inner nuclear membrane (~20%) (Fig. 5B) whereas their presence in nucleoplasm-associated structures was greater than that for type-1 IP_3R, reaching 54%. Nearly 26% of particles accumulated in the nucleolus (Fig. 5B, inset). Here the particles were also found in clusters, reaching 78% compared to isolated particles; the mean number of particles within a given cluster was higher for type-3 than for type-1 receptors. The outer nuclear membrane was not labeled.

In parallel control experiments without the primary antibody, virtually no gold particles were found (not shown), further demonstrating the specificity of the immunogold labeling.

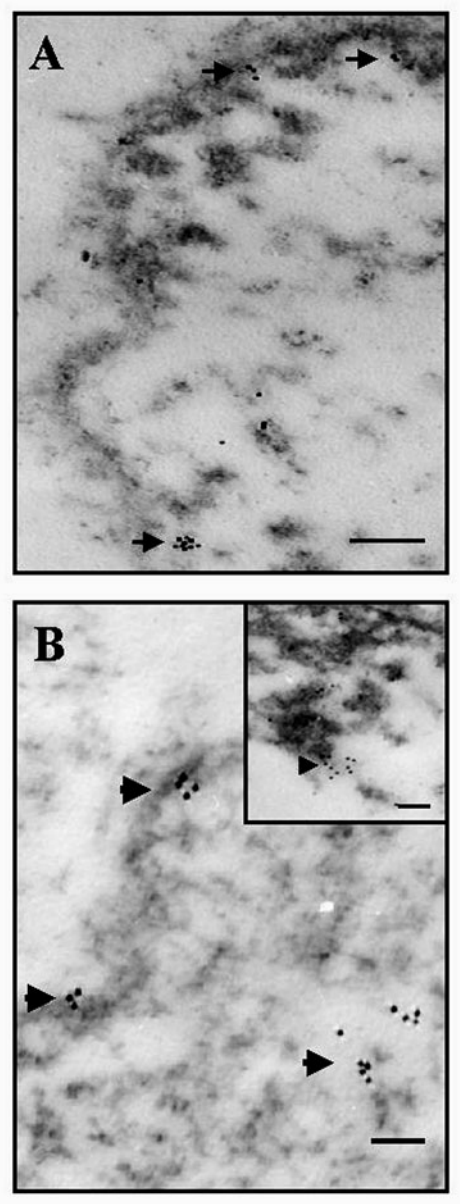

Fig. 5. Immunogold localization of IP_3 receptors in isolated nuclei. Isolated nuclei from skeletal muscle cells were immunolabeled for type-1 or type-3 IP_3R with isoform-specific antibodies linked to 10 nm gold particles. (A) Type-1 IP_3R gold particles (indicated by arrows) located in the nuclear membrane mainly grouped in clusters. (B) Type-3 IP_3R labeling (indicated by arrows) is located in groups of particles in the nuclear membrane and the nucleoplasm. Inset, the nucleolus presents clusters associated with undefined structures. Bar, 100 nm.

Changes in nucleoplasmic Ca^{2+} induced by IP_3Rs

Nuclei were loaded with Fluo-3/AM to determine whether IP_3Rs present in the nucleus could directly induce nuclear Ca^{2+} increases. For each experiment, the nuclear envelope was initially loaded with Ca^{2+} in the presence of 1-2 mM ATP and 100-200 nM extranuclear Ca^{2+}, as described (Gerasimenko et al., 1995); nuclei were then exposed to IP_3 in the absence of Ca^{2+}. Batch application of 10 μM IP_3 elicited a quick and transient increase of Ca^{2+}, as shown in the response of a nucleus (Fig. 6A, right panel), representative of 40 different nuclei analyzed from eight different preparations. In order to determine the specificity of this signal, nuclei from the same preparation were either stimulated with saline, or were pre-incubated with either 50 μM 2-APB, an inhibitor of the IP_3-dependent signals, or 10 μM xestospongin B (XeB), a competitive blocker of IP_3Rs (Jaimovich et al., 2005). In all cases, no response was obtained (n=25 nuclei from different preparations, for both the vehicle and 2-APB; n=18 nuclei from two different preparations for XeB). In all studies using either saline or inhibitor, the response of the nuclei was first proved positive with IP_3 (Fig. 6A, left panel). In addition, we used isolated nuclei from the 1B5 cell line, characterized by the lack of expression of RyR isoform, in which myotubes responded to depolarization with an increase in nuclear Ca^{2+} that was inhibited by 2-APB (Estrada et al., 2001). Therefore, we stimulated isolated nuclei from IB5 cells with 10 μM IP_3 and, as for primary cells; we obtained a similar, quick and transient response, which was inhibited by both 50 μM 2-APB and 10 μM XeB (Fig. 6B). Again, nuclei treated with saline showed no response (Fig. 6B, n=35 for IP_3; n=25 for both the vehicle and 2-APB; n=15 for XeB).

In order to ascertain the origin of Ca^{2+}, we studied fluorescence in a nucleus suspension and, instead of fluo-3/AM, we used dyes that have been reported to specifically locate to either the nuclear envelope (mag-fluo-4) or the nucleoplasm (fluo-4 dextran) (Gerasimenko et al., 1995; Gerasimenko et al., 2003). When stimulated with 10 μM IP_3, nuclei loaded with mag-fluo-4 showed a quick decrease of fluorescence (Fig. 6C) that corresponds to a decrease in Ca^{2+} in the nuclear envelope (representative trace of 20 different measurements from four different preparations), whereas when stimulated with 10 μM IP_3 the nuclei loaded with fluo-4

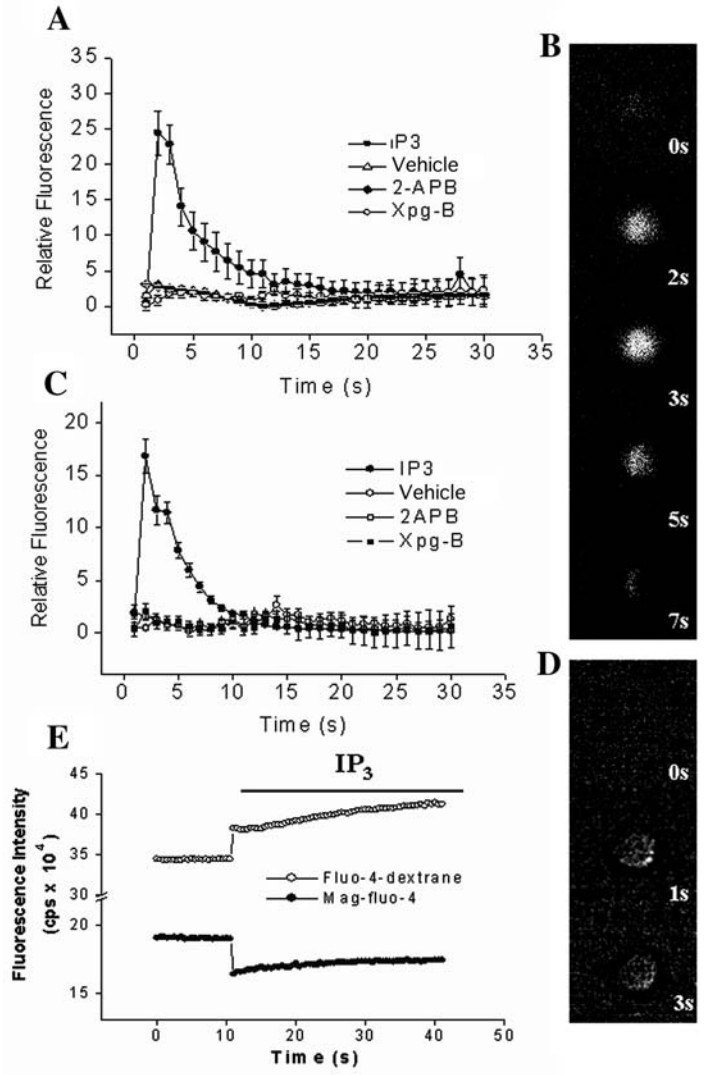

Fig. 6. IP_3 induced nucleoplasmic Ca^{2+} increases in isolated myonuclei. The time course of relative fluorescence change (ratio between the fluorescent difference, stimulated minus basal and the basal value) as a function of time is shown. (A) Stimulation of isolated myonuclei with 10 μM IP_3 caused a quick and transient increase in calcium (●). Such an increase was inhibited by 2-APB (■) and XeB (○). The physiological solution (vehicle) alone did not induce any signal (△). (B) Series of fluo-3 fluorescent images of myonuclei recorded at the times indicated. Basal fluorescence is shown in the top, the following image was taken immediately after 10 μM IP_3 was added. (C) Isolated nuclei from 1B5 myotubes, stimulated with 10 μM IP_3 display a quick and transient increase in calcium (●), that was inhibited by 2-APB (□) and XeB (■). The vehicle IP_3 alone did not induce a signal (○). (D) Series of fluo-3 fluorescent images in 1B5 nuclei collected at the times indicated. Basal fluorescence is shown at the top, the images that follow were taken after the stimulus with 10 μM IP_3. (E) Fluorescence in a nucleus suspension using dyes reported to specifically locate to either the nuclear envelope (mag-fluo-4) or the nucleoplasm (fluo-4 dextran). The suspension was stimulated with 10 μM IP_3 as indicated by the bar, traces shown are representative of 20 different measures from four different preparations. Nuclear diameter is approximately 10 μm.

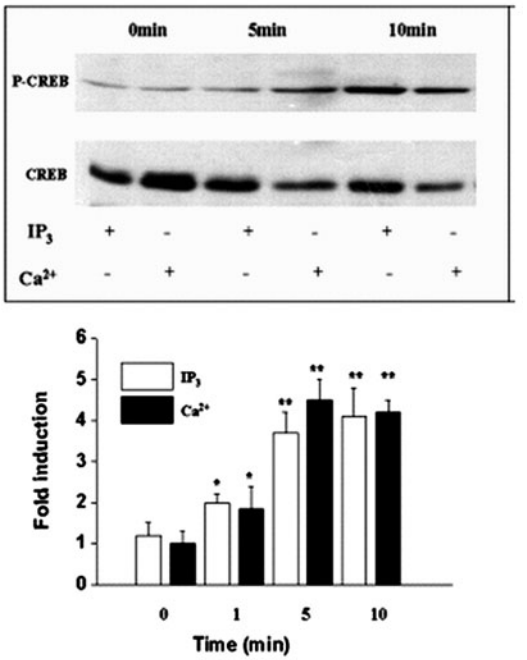

Fig. 7. IP_3 and calcium induced CREB phosphorylation in isolated myonuclei. Top panel, western-blots of CREB phosphorylation at Ser133 following additions of Ca^{2+} and IP_3 solutions at the times indicated. Incubation conditions were similar to those described for Fig. 6C. To correct for loading, blots were probed with an antibody that recognizes the phosphorylated and non-phosphorylated forms of CREB. Bottom panel, fold induction (mean±s.e.m. of three different experiments) of CREB phosphorylation over control levels. Significant differences $*P<0.05$ and $**P<0.01$ in induction of phosphorylation were observed between control and IP_3- and Ca^{2+}-treated groups at the indicated time points.

dextran (Fig. 6C) showed a quick increase in fluorescence that corresponded to an increase in nucleoplasmic Ca^{2+} (representative trace of 20 measures from four different preparations). Both mag-fluo-4 and fluo-4 dextran signals were inhibited in the presence of XeB (not shown). It is worth noting that the kinetics of Ca^{2+} mobilization were similar for all three dyes used (compare with Fig. 6A,B).

Stimulation of nuclear IP_3R-induced CREB phosphorylation

We have previously demonstrated that the Ca^{2+} increase induced by membrane depolarization stimulates CREB phosphorylation (Powell et al., 2001), and also determined that inhibitors of nuclear slow Ca^{2+} signals inhibited CREB phosphorylation (Carrasco et al., 2003). Although those experiments were performed in intact myotubes, we reasoned that if isolated nuclei could induce a Ca^{2+} increase, and considering that they have protein kinases (PKA, CAMK and PKC) (Santella and Carafoli, 1997), it was therefore important to look for an increase in CREB phosphorylation in this system. To test this hypothesis, isolated nuclei were stimulated with 10 μM IP_3 for various times, lyzed, separated in 10% SDS-polyacrylamide gels and blotted with a specific antibody against the phosphorylated form of CREB. Interestingly, IP_3 induced a rapid increase in CREB phosphorylation in isolated

nuclei to about twofold above basal levels in the first minute, reaching more than threefold greater than basal levels after 5 and 10 minutes (Fig. 7). We also tested whether Ca^{2+} alone stimulated CREB phosphorylation in isolated nuclei. To this aim, we stimulated the cells with 1 μM $CaCl_2$ and found an increase in CREB phosphorylation that reached a maximum (three to fourfold over basal) at 5 minutes (Fig. 7B, *n*=5 independent experiments).

Discussion

Ca^{2+} release induced by depolarization of skeletal muscle cells can be separated into two independent components; the fast Ca^{2+} transient, homogenously distributed through the cell that corresponds to excitation-contraction coupling, and the slow Ca^{2+} transient, with a distinct nuclear component, generated by IP_3 (Jaimovich et al., 2000). Slow Ca^{2+} signals with similar characteristics, although displaying particular kinetics can also be elicited by androgens like testosterone (Estrada et al., 2000; Estrada et al., 2003), or hormones such as insulin-like growth factor (Espinosa et al., 2004). Slow Ca^{2+} signals have been shown to participate in phosphorylation of both MAP kinases ERK 1 and 2, and transcription factor CREB, as well as in the expression of early genes *fos*, *jun* and *egr-1* (Powell et al., 2001; Carrasco et al., 2003). In this work, we determined that type 1 and 3 IP_3Rs are expressed and are functional in myonuclei, mediating nucleoplasmic Ca^{2+} changes, which in turn induce CREB phosphorylation.

Several reports indicate the presence of active IP_3R in the nucleus. Electrophysiological measurements directly show IP_3-dependent receptor channel activity in isolated nuclei from *Xenopus laevis* oocytes (Stehno-Bittel et al., 1995), or from the mammalian nuclear envelope (Boehning et al., 2001). On the other hand, Gerasimenko and co-workers (Gerasimenko et al., 1995), elegantly demonstrated using confocal microscopy that isolated nuclei from liver show an increase of nucleoplasm Ca^{2+} after IP_3 stimulation, and determined that the source of Ca^{2+} was in the nuclear envelope. However, the source of nuclear Ca^{2+} remains a controversial issue. The presence of nuclear pores, with a diameter of 9 nm and a length of 15 nm, suggest that it should be permeable to all molecules up to 50 kDa (Stoffler et al., 1999), suggesting that Ca^{2+} is able to equilibrate rapidly between the cytosolic and nuclear compartments by simple diffusion. In neuroendocrine pancreatic β-cells it was demonstrated that cytosolic Ca^{2+} oscillations induced by glucose, potassium and carbachol enter the nucleus without restriction (Brown et al., 1997). No significant differences between nuclear and cytosolic Ca^{2+} concentration were observed in HeLa cells when monitoring Ca^{2+} inside the nucleus using a chimeric cDNA encoding a fusion protein with the photoprotein aequorin, and a nuclear translocation signal derived from the rat glucocorticoid receptor (Brini et al., 1993).

However, it has been demonstrated both in oocytes and in HeLa cells that it is possible to induce an IP_3-dependent rise of nuclear Ca^{2+}, independently of the cytoplasm (Lui et al., 1998; Santella et al., 1998). The participation of IP_3Rs in regulating nuclear Ca^{2+} in skeletal muscle has not yet been described.

As for the distribution of IP_3Rs in the nucleus, not much is known about their precise topology. Humbert and colleagues (Humbert et al., 1996) demonstrated that IP_3Rs are located in the inner nuclear membrane. However, it has been recently demonstrated that different IP_3R isoforms have different locations in the nucleus; in HepG2 liver cells, type-2 IP_3Rs are expressed in the nucleus, mainly in the nuclear envelope and occasionally in the nucleoplasm, whereas the type-3 IP_3R was only expressed in the cytoplasm, and type-1 was absent in these cells (Leite et al., 2003). In addition, different IP_3-induced Ca^{2+} responses were measured in the nucleus and cytoplasm, which were attributed to different characteristics of the IP_3R isoforms (Leite et al., 2003). In endothelial cells, type-2 IP_3R are distributed uniformly within the nucleus, but type-1 and type-3 IP_3Rs were absent from the nucleus (Laflamme et al., 2002). In neonatal rat cardiomyocytes, the distribution of receptors is similar to that presented here for myotubes (Ibarra et al., 2004), whereas in adult cardiac cells, type 2 IP_3R is present in the nuclear envelope (Bare et al., 2005). It is interesting to note that even though there is continuity between the ER and nuclear envelope membranes, their protein composition appears to be different. The ER itself is not uniform with respect to channel, pump and protein distribution (reviewed by Petersen et al., 2001); for example, calsequestrin was located in the ER lumen and was enriched within small vacuoles that were also equipped with SERCAs (sarco/endoplasmic reticular Ca^{2+}-ATPases). Some, but not all, of these vacuoles (calciosomes) contained IP_3Rs (Volpe et al., 1991). Our results showing that isolated nuclei lack sarcoplasmic reticulum markers confirm this point.

The presence of IP_3R in the nucleoplasm is a controversial point because it is assumed that the receptor will necessarily be associated with a membrane. The presence of tubular structures inside the nucleus was described in the C6 glioma cell line and in HeLa cells. These structures apparently correspond to extrusions of the nuclear envelope, enriched in IP_3R (Lui et al., 1998; Lui et al., 2003). In isolated nuclei from *Xenopus* oocytes, expression of lamin B1 and B2, was demonstrated to induce formation of intranuclear membranes that resemble ER cisternae (Ralle et al., 2004).

The nucleoplasmic reticulum, a reticular network of nuclear Ca^{2+} store that is continuous with the ER and nuclear envelope was recently proposed in SKHep1 epithelial cells (Echevarria et al., 2003) to be enriched in type-2 IP_3R and to a lesser extent in type-3 IP_3R. Moreover IP_3-induced Ca^{2+} release from the nucleoplasmic reticulum was detected by confocal laser-scanning microscopy examining the release of nitrophenylethyl ester-caged IP_3 microinjected into the cell (Echevarria et al., 2003). In the present study, we found that in skeletal muscle cells all three isoforms of IP_3R are expressed with different intracellular distribution. Type-2 IP_3R was distributed more or less homogeneously with a reticular pattern that probably represents sarcoplasmic reticulum membranes in undifferentiated myotubes. We have previously reported that type-1 IP_3R is expressed in the nuclear envelope (as was now confirmed by its colocalization with LAP2, the inner nuclear membrane marker, and by the location of immunogold particles), as well as in the sarcoplasmic reticulum with a striated pattern (Powell et al., 2001). Furthermore, type-3 IP_3R was mainly distributed inside the nucleus, not exclusively associated with the nuclear membrane, as assessed both by minimal confocal colocalization using LAP2, and by electron microscopy using

inmunogold. This suggests that most type-3 (and also a number of type-1) IP_3Rs are probably located in a different structure; it is possible that some anti-IP_3R antibodies would bind to nuclear proteins other than the receptor (Bare et al., 2005). Indeed the western blot does show very faint bands in addition to that of ~260 kDa, which corresponds to the receptor. An additional protein having the same molecular size of the receptor is also a possibility. Non-specific binding is unlikely as we tried four different antibodies raised against two different peptides and obtained similar results. We can conclude then that either a membrane structure, possibly the recently described nucleoplasmic reticulum (Echevarria et al., 2003) is present in our nuclear preparation and we are unable to visualize it under EM, or that there is specific binding of the antibody to an unknown nuclear protein that we cannot identify. Analysis of $[^3H]IP_3$ binding experiments reflect the important component of total receptors located in the nuclear fraction (Fig. 4). Taken together, these data show that IP_3R isoforms have a differential intracellular distribution in skeletal muscle cells, and that at least one of these isoforms functions as a Ca^{2+} release channel in nuclear compartments. This finding, together with the presence of Ca^{2+}-ATPase activity associated to the nuclear fraction, suggests a role of the nuclear envelope and associated membrane structures in the regulation of nucleoplasmic Ca^{2+} concentration.

We also provide here direct evidence for the control of CREB phosphorylation by the increase in nuclear Ca^{2+}. We found that IP_3 induced quick CREB phosphorylation in isolated myonuclei. As for the protein responsible for CREB phosphorylation, we recently demonstrated that PKC-α is present in the nucleus in basal conditions, which also translocates to the nucleus after depolarization of myotubes. We also showed that PKC-α is responsible for CREB phosphorylation induced by IP_3 (Cardenas et al., 2004). Thus, PKC appears to be a good candidate for directly or non-directly controlling CREB phosphorylation, although further studies are needed in order to confirm this point.

Local nuclear IP_3 production and local Ca^{2+} release are likely to play a major role in regulating both transcription factors and the transcription process of many genes; isolated nuclei from cultured muscle cells constitute a good model system to unravel such mechanisms.

C.C. was supported by a graduate student fellowship from the Comisión Nacional de Investigación Científica y Tecnológica (CONICYT), Programa de Mejoramiento de la Calidad y la Equidad de la Educacion Superior UCH9903, and the Association Française contre les Myopathies (AFM). This work was supported by Fondo de Investigación Avanzada en Areas Prioritarias, Grant 15010006 (to E.J.), the Direction des Systèmes de Forces et de la Prospective (grant 026065093) and the AFM (to J.M.). This work was made possible, in part by the ECOS Sud-CONYCIT exchange program (C03S02).

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