Novel biochemical and functional insights into nuclear Ca\textsuperscript{2+} transport through IP\textsubscript{3}R and RyRs in osteoblasts

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Adebanjo, Olubengwa A., Gopa Biswas, Baljit S. Moonga, Hindupur K. Anandatheerthavarada, Li Sun, Peter J. R. Bevis, Bali R. Sodam, F. Anthony Lai, Narayan G. Avadhani, and Mone Zaidi. Novel biochemical and functional insights into nuclear Ca\textsuperscript{2+} transport through IP\textsubscript{3}R and RyRs in osteoblasts. Am J Physiol Renal Physiol 278: F784–F791, 2000.—We report the first biochemical and functional characterization of inositol trisphosphate receptors (IP\textsubscript{3}R) and ryanodine receptors (RyRs) in the nuclear membrane of bone-forming (MC3T3-E1) osteoblasts. Intact nuclei fluoresced intensely with anti-RyR (Ab\textsubscript{34}) and anti-IP\textsubscript{3}R (Ab\textsubscript{30}) antisera in a typically peripheral nuclear membrane pattern. Isolated nuclear membranes were next subjected to SDS-PAGE and blotted with isospecific-specific anti-receptor antiser, notably Ab\textsubscript{40}, anti-RyR-1, anti-RyR-2 (Ab\textsubscript{290}), and anti-RyR-3 (Ab\textsubscript{180}). Only anti-RyR-1 and Ab\textsubscript{40} showed bands corresponding, respectively, to full-length RyR-1 (~500 kDa) and IP\textsubscript{3}R-1 (~250 kDa). Band intensity was reduced by just ~20% after brief tryptic proteolysis of intact nuclei; this confirmed that isolated nuclear membranes were mostly free of endoplasmic reticular contaminants. Finally, the nuceloplasmic Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{np}) was measured in single nuclei by using fura-dextran. The nuclear envelope was initially loaded with Ca\textsuperscript{2+} via Ca\textsuperscript{2+}-ATPase activation (1 mM ATP and ~100 nM Ca\textsuperscript{2+}). Adequate Ca\textsuperscript{2+} loading was next confirmed by imaging the nuclear envelope (and nucleoplasm). Exposure of Ca\textsuperscript{2+}-loaded nuclei to IP\textsubscript{3} or cADP ribose resulted in a rapid and sustained [Ca\textsuperscript{2+}]\textsubscript{np} elevation. Taken together, the results provide complementary evidence for nuceloplasmic Ca\textsuperscript{2+} influx in osteoblasts through nuclear membrane-resident IP\textsubscript{3}Rs and RyRs. Our findings may conceivably explain the direct regulation of osteoblastic gene expression by hormones that use the IP\textsubscript{3}-Ca\textsuperscript{2+} pathway.

nuclear calcium channels; bone formation; osteoblasts; osteoporosis; ryanodine receptors; inositol 1,4,5-trisphosphate receptors

CELLOLAR FUNCTIONS, such as differentiation, growth, and metabolism, are all regulated by cytoplasmic Ca\textsuperscript{2+} signals generated in response to membrane receptor activation. It has long been speculated, and upheld more recently, that such cytosolic Ca\textsuperscript{2+} changes are translated directly into nuclear Ca\textsuperscript{2+} changes through nuclear pores (2, 6). Nevertheless, it is unclear whether such passive Ca\textsuperscript{2+} influx could, in fact, regulate nuclear functions as critical as DNA repair, troposimerase activation, polymerase unfolding, gene transcription, and apoptosis (41). Several recent studies indicate that despite the presence of nuclear pores, there is an active Ca\textsuperscript{2+} gradient across the nuclear membrane (3, 9). Furthermore, nuclear pores can apparently exist in Ca\textsuperscript{2+}-permeable and Ca\textsuperscript{2+}-impermeable states depending on the peri-nuclear space Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{space}) (38). The latter appears to be monitored by an EF-hand-containing protein of the nuclear pore complex (42). Recent confocal microscopic studies have shown nuclear Ca\textsuperscript{2+} waves to emanate at the cytosol-nucleus border, indicating again, a relatively Ca\textsuperscript{2+}-impearent nuclear membrane (9). Indeed, it has been known for over three decades from studies demonstrating high impedance of the nuclear envelope, that its ionic permeability was tightly regulated (26).

Being an extension of the endoplasmic reticulum (ER), the nuclear envelope is now regarded as the equivalent of a “Ca\textsuperscript{2+} store.” Thus most ER Ca\textsuperscript{2+} transporters appear in the nuclear membrane (41). Resident in the outer, and presumably in the inner, nuclear membrane is a Ca\textsuperscript{2+}-ATPase (11, 15, 24, 35). The latter shares most properties with its ER counterpart (24). Conceivably, its function is to permit active nuclear envelope filling against a Ca\textsuperscript{2+} gradient. In addition, there is evidence for nuclear membrane Ca\textsuperscript{2+} release channels of the inositol 1,4,5-trisphosphate receptor (IP\textsubscript{3}R) type (11, 21, 27, 29, 36). Nevertheless, biochemical, immunological, and functional evidence for ryanodine receptors (RyRs) on the nuclear membrane is relatively scant. Furthermore, several IP\textsubscript{3} generating molecules, including phosphatidylinositol (PI) kinases, PI transfer proteins, and phospholipases are located within distinct nuclear subcompartments. Although the latter findings are suggestive of intra-nuclear IP\textsubscript{3} generation (5, 7, 37), it is unclear whether the RyR agonist cADP ribose (cADPr) can also be generated within the nucleoplasm.

Here we report studies on the osteoblast, a cell that is exquisitely sensitive not only to bone-forming hor-
mones but also to Ca\(^{2+}\) to which it is exposed locally, presumably during active mineralization. The triggering of various hormone receptors, such as the parathyroid hormone receptor (17), results in IP\(_3\) generation and Ca\(^{2+}\) release through microsomal IP\(_3\)Rs. It is therefore conceivable that the cytosolically generated IP\(_3\) also triggers putative nuclear membrane IP\(_3\)Rs. Furthermore, as both IP\(_3\)Rs and RyRs have high-affinity Ca\(^{2+}\)-binding domains, any rise in cytosolic Ca\(^{2+}\) could, in principle, activate both channels. The possibility then, that nuclear Ca\(^{2+}\) might regulate gene expression, and hence, bone formation, prompted our present studies into osteoblastic nuclear Ca\(^{2+}\) transport. We provide complementary lines of evidence, biochemical and functional, for nucleoplasmic Ca\(^{2+}\) influx through nuclear membrane-resident, type I isoforms, of the IP\(_3\)R and RyR families. This represents the first evidence for a nuclear Ca\(^{2+}\) channel in any bone cell.

**MATERIALS AND METHODS**

Materials and buffers. Fura 2, fura 2-acetoxyethyl ester, and fura-dextran were purchased from Molecular Probes (Eugene, OR). Tissue culture materials, including heat-inactivated FBS were bought from Gibco-BRL (Gathersburg, MD). ETGA, ionomycin, ATP, IP\(_3\), and cADPR were all obtained from Sigma Chemical (St. Louis, MO).

Osteoblast culture and nuclear isolation. MC3T3-E1 osteoblastic cells were grown in HEPES-buffered RPMI-1640, supplemented with FBS (10%, vol/vol), glutamine (1%, wt/vol), penicillin (50 KU/l), and streptomycin (50 g/l). The cells were subcultured at confluence by washing in EDTA, followed by gentle trypsinization (0.025%, wt/vol; 2 min), addition of RPMI-1640 before centrifugation, and resuspension in medium. The cells were maintained in tissue culture flasks (37°C; Fisher Scientific, St. Louis MO) and harvested in their logarithmic growth phase.

For isolation of nuclei, the cells were scraped at confluence and suspended in cold TKM-sucrose solution (Tris·HCl, with 25 mM KCl, 5 mM MgCl\(_2\), and 0.25 mM sucrose; pH = 7.5). They were homogenized in a glass homogenizer (×9), filtered through three layers of sterile gauze, and then centrifuged (at 700 g, 10 min) to pellet the nuclei. The nuclear pellet was resuspended in TKM-sucrose solution (20 mM), rehomogenized (×5), and then centrifuged (700 g, 10 min). The resulting pellet was finally suspended and centrifuged again (1,000 g, 5 min) to produce a highly purified preparation of intact nuclei (all procedures, 4°C). In separate experiments, higher nuclear purity was obtained by using the methods of Malviya et al. (29) and Humbert et al. (21), involving use of a higher sucrose concentration of 2.2 M.

Immunonucleochemical and confocal microscopic studies. Immunonucleochemical studies used two anti-receptor antisera: Ab\(^{38}\), raised to the consensus calmodulin-binding RyR sequence, and Ab\(^{40}\), raised to the purified IP\(_3\)R-1 protein (Table 1) (44). Coverslips (360 mm\(^2\), Fisher, St. Louis, MO) containing freshly isolated osteoblastic nuclei were first incubated with normal goat serum in multiwell dishes (in 10 mM PBS, 1:10, pH = 7.4, 15 min). Excess serum was removed and replaced with Hanks’ balanced salt solution (HBSS; Gibco-BRL). The nuclei were then incubated without antibody, or with nonimmune rabbit serum (control), Ab\(^{34}\), or Ab\(^{40}\) (in HBSS, 1:100, vol/vol, 1 h). The coverslips were then rinsed gently with HBSS, drained, incubated with goat anti-rabbit FITC (Sigma Chemical, in HBSS, 1:100; 1 h), washed gently, and drained. For epifluorescence microscopy, a 510-nm FITC filter was positioned in the emission path of the microspectrofluorimeter (Diaphot, Nikon, Tokyo, Japan). In addition, coverslips mounted onto glass slides were viewed in a laser confocal scanning microscope (Leica, Deerfield, IL). Fluorescent nuclei were visualized, using volume element imaging, as serial, 1-μm-thick optical sections in the coronal plane of each nucleus (44).

Membrane isolation, protease protection assay, and Western blotting. For the isolation of ER membranes, intact osteoblasts were homogenized in sucrose-mannitol buffer (in mM) 20 HEPES, 70 sucrose, 220 mannitol, 2 EDTA, and 0.1 phenylmethylsulfonylfluoride, as well as 1.25 μg/ml each of antipain, chymostatin, leupeptin, and pepstatin. The homogenate was centrifuged (15,000 g, 20 min) to remove mitochondrial and nuclear membrane fractions. The supernatant was then centrifuged (10,000 g, 1 h, 4°C) to obtain the ER membrane fraction. The latter was suspended in homogenization buffer and repelleted (100,000 g, 1 h). The final pellet was resuspended in 500 μl sucrose-mannitol buffer (as described above) and stored at −70°C.

For isolation of purified nuclear membranes, the isolated nuclei (see above) were suspended in sucrose-mannitol buffer and centrifuged (100,000 g, 30 min). The pellet was then resuspended in the same buffer and sonicated (1 min). In separate experiments, nuclear and ER membranes were subjected to brief trypsin treatment (120 μg/mg protein) in a 500-μl reaction volume (on ice, 30 min) (4). The reaction was stopped by the addition of a trypsin inhibitor (1.2 mg/mg protein). Nuclear membranes were then isolated as described above. The trypsin-treated ER membranes were used as such.

The isolated ER and nuclear membranes were next incubated with 3 × Laemmli's sample buffer (37°C, 20 min). Two hundred micrograms of protein (estimated by Lowry's method) was then separated by SDS-PAGE (6%; Bio-Rad Minigels), followed by electroblotting onto nitrocellulose membranes. The blots were then air-dried and blocked with PBS (0.3%, vol/vol; 1 h), followed by incubation with Ab\(^{38}\), anti-RyR-1, Ab\(^{29}\), or Ab\(^{30}\) (in PBS 0.05%, vol/vol; 1:3,000; 1 h). The immunoblots were then developed with horseradish peroxidase-conjugated anti-rabbit antibodies (in PBS 0.05%, vol/vol; 1:30,000) by using Pierce's Supersignal Ultra substrate solution, per manufacturer's protocol. The blots were then quantitated by using the Bio-Rad Multianalyst program.

Nuclear Ca\(^{2+}\) measurements. The method used was modified from that used by Gerasimenko et al. (11). Isolated intact
nuclei were incubated with fura-dextran (20 µM, 45 min, 4°C) in 12 ml "standard" solution comprising (in mM) 125 mM KCl, 2 mM K2HPO4, 50 mM HEPES, 4 mM MgCl2, 0.1 mM EGTA, and 1 mM ATP (pH = 7.4) ([Ca2+] < 5 nM, by fura-2-based cuvette measurements). The dye-loaded nuclei were washed in standard solution and centrifuged (1,000 g, 1 min). They were then seeded, at a low density, on glass coverslips (360 mm², Fisher) for photometric studies. The nuclei were allowed to settle for 15 min in standard solution. The nuclear envelope was then loaded with Ca2+ in the same buffer (30 min, 37°C) at an extranuclear [Ca2+] of 200 nM (obtained by adding 107 µl of 1 M CaCl2 to 50 ml modified standard solution with 10 mM EGTA) (43). The relatively low extranuclear [Ca2+] in the presence of ATP permitted active nuclear envelope Ca2+ loading, presumably through an activated Ca2+-ATPase. Experiments performed at either higher (500 nM) or lower (< 5 nM) extranuclear [Ca2+] did not produce effective nuclear envelope Ca2+ loading (data not shown).

All photometric nucleoplasmic Ca2+ concentration ([Ca2+]n) measurements were done in the standard solution but without added ATP (see above). In addition, fast kinetic [Ca2+]n measurements were made every 250 ms by using an Ion Optix microspectrofluorimeter and fura-dextran as the fluorescent indicator (43). Nuclei were exposed to excitation λs of 340 and 380 nm, and the emitted fluorescence was monitored at 510 nm. Photon counts per second were used to calculate the emitted intensity ratio, I405/I480. Finally, the fluorochrome was calibrated (43) (see RESULTS).

RESULTS

Nuclear membrane immunolocalization of IP3R and RyR. We first examined whether IP3Rs and RyRs were detectable immunocytochemically by using our highly specific anti-receptor antisera Ab40 and Ab34. Note from Table 1 that Ab40 detects the most abundantly expressed IP3R-1 isoform (44). In contrast, having being raised against the consensus calmodulin-binding RyR sequence, Ab34 does not distinguish between the three RyR isoforms (44). Intact, nonpermeabilized, osteoblastic nuclei fluoresced intensely with both the antisera, Ab40 or Ab34 (Fig. 1, B and D). Nuclei incubated without antisera (Fig. 1A) or with nonimmune rabbit serum (Fig. 1C) did not stain.

Confocal microscopy indicated that the IP3Rs and RyR fluorescence was almost strictly peripheral, with virtually no nucleoplasmic staining (Figs. 2, A and B). The confocal microscopic data were further analyzed by volume element imaging, which allowed a three-dimensional view of each confocal section. This again confirmed a peripheral nuclear membrane localization of the immunofluorescence.

Osteoblastic expression of type 1 RyR and type 1 IP3R isoforms. To examine for the presence of specific nuclear membrane IP3R and RyR isoforms, we performed SDS-PAGE and Western blotting by using our repertoire of isoform-specific, anti-IP3R and RyR antisera, namely, Ab40, anti-RyR-1, Ab129, and Ab180 (Table 1). Of note is that anti-RyR-1 was raised to the purified RyR-1 protein, whereas Ab129 and Ab180 were raised to unique peptide sequences of RyR-2 and RyR-3, respectively (Table 1). That these antisera specifically detect the respective proteins has been demonstrated previously (44). However, we again confirmed antibody specificity by using ER membranes from skeletal muscle (RyR-1 and RyR-3) and brain (RyR-2 and RyR-3) (Fig. 3). Similarly, immunoblotting of osteoblastic nuclear mem-

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**Fig. 1.** Intense immunofluorescent staining of isolated, intact, nonpermeabilized osteoblastic nuclei with highly specific antibodies to inositol 1,4,5-trisphosphate receptor (IP3R; Ab40; B) or ryanodine receptor (RyR; Ab34; A). Control nuclei incubated without antiserum (A) or with nonimmune rabbit serum (C) showed no appreciable fluorescence. Experiments were repeated 5 times; each time up to 20 nuclei were visualized.
branes revealed bands corresponding to the full-length IP₃R-1 (∼270 kDa) and RyR-1 (∼500 kDa) (Fig. 3). No bands were observed with Ab₁₂₉ and Ab₁₈₀, excluding significant expression of RyR-2 and RyR-3. In parallel, osteoblastic ER membranes also expressed the same IP₃R and RyR isoform, as did the nuclear membrane, notably the type 1 isoforms (Fig. 3). Although the presence of IP₃Rs on the nuclear membrane is consistent with the studies of Guihard et al. (15), the group did not detect any RyR isoforms, including, RyR-1.

A possibility arises, however, that our isolated nuclear preparations were contaminated with ER membranes and that these, rather than purely nuclear membranes, contributed to the detected signals. To check for such contamination, we used two complementary approaches. First, we blotted isolated nuclear membranes with an antibody to lamin-B, an inner nuclear membrane protein, as well as NADPH cytochrome P-450 reductase, an ER marker enzyme. We found lamin-B bands but no immunoreactivity for NADPH cytochrome P-450 reductase in our nuclear preparations. The converse was true for our ER preparations (not shown). These results suggested that the nuclear membrane preparations were mostly free from significant ER contamination.

Next, we incubated intact nuclei and ER membranes, in parallel, with trypsin (120 µg/mg protein, 30 min, on ice). The rationale of these experiments was that any exposed receptors, which would include those on attached ER fragments, would be amenable to proteolysis by trypsin. In contrast, receptors that were present intranuclearly, for example, in the inner nuclear membrane, would not be accessible to trypsin and hence would be insensitive to protease degradation. Thus, in the absence of ER contamination, nuclear membrane band intensity would be expected not to diminish significantly on proteolysis of intact nuclei. In contrast, trypsin should proteolyze any ER membrane fragments, resulting in a significant reduction in signal intensity. We found that nuclear membrane band intensity fell only by ∼20% after brief tryptic proteolysis of intact nuclei (Fig. 4). However, when ER membranes were treated similarly, there was an expected reduction in band intensity by ∼80% (Fig. 4). Taken together, the experiments confirm that our isolated nuclear preparations were mostly free of significant ER contamination; this makes it unlikely that the nuclear membrane IP₃R-1 and RyR-1 bands were derived solely from

Fig. 2. Confocal microscopic localization of IP₃Rs and RyRs to osteoblast nuclear membrane. A and B: intense peripheral immunofluorescent staining, respectively, with highly specific anti-IP₃R (Ab₄⁰) and anti-RyR (Ab₃⁴) antisera. Serial sections were obtained in coronal plane of each nucleus at 1-µm intervals and analyzed by volume element imaging. There is a typically peripheral nuclear membrane fluorescence with minimal nucleoplasmic staining. Experiments were repeated 2 times; each time up to 20 nuclei were visualized.

Fig. 3. Osteoblast nuclear (nuclei) and endoplasmic reticular (ER) membranes, as well as ER membranes from skeletal muscle (SkM) and brain immunoblotted with a panel of isoform-specific antibodies to IP₃R (∼250 kDa) and RyR (∼500 kDa). These include anti-IP₃R-1 (Ab₄⁰; top left), anti-RyR-1 (top right), anti-RyR-2 (Ab₁₂₉; bottom left), and anti-RyR-3 (Ab₁₈₀; bottom right). Their specificities are detailed in Table 1 and Ref. 44. Experiments were repeated twice. Mr, marker.
contaminating ER fragments. The experiments also indicate a possible inner nuclear membrane of these receptors. A similar approach was used successfully by Anandatheerthavarada et al. (4).

Taken together, the absence of an ER marker, NADPH cytochrome P-450 reductase, from our nuclear preparations together with the retention of IP3R-1 and RyR-1 bands on trypsin proteolysis of intact nuclei strongly suggest a lack of significant ER contamination and attest to the purity of these preparations.

Nucleoplasmic Ca\textsuperscript{2+} influx through RyRs and IP3Rs. Fura-dextran-based fast kinetic [Ca\textsuperscript{2+}]\textsubscript{np} measurements were made photometrically (see MATERIALS AND METHODS). We initially calibrated fura 2 by using a modified “intracellular” protocol. Briefly, fura 2-loaded nuclei were bathed in a “calibration” solution consisting of (in mM) 125 KCl, 2 K\textsubscript{2}HPO\textsubscript{4}, 5 glucose, 10 HEPE\textsubscript{5}, 0.8 MgCl\textsubscript{2}, and 1 CaCl\textsubscript{2}. The Ca\textsuperscript{2+} ionophore ionomycin (20 µM) was then applied to obtain the due to highest cytosolic [Ca\textsuperscript{2+}] (\textit{R}_{\text{max}}) and the minimum fluorescence intensity at 380 nm (\textit{F}_{\text{min}}) (Fig. 5, A and B). Extraneur nuclear Ca\textsuperscript{2+} was then chelated by adding 1.2 mM EGTA to obtain values of the minimum ratio (\textit{R}_{\text{min}}) and maximal fluorescence intensity at 380 nm (\textit{F}_{\text{max}}). The dissociation constant (\textit{K}_d) for Ca\textsuperscript{2+} and fura 2 at a temperature of 20°C, an ionic strength of 0.1 M, and a pH of 6.85, is 224 nM. The values were substituted into the equation \[ [\text{Ca}^{2+}] = K_d \times \frac{(R - R_{\text{min}})/(R_{\text{max}} - R)}{\left(F_{\text{max}}/F_{\text{min}}\right)} \]. A calibration curve was essentially linear between Ca\textsuperscript{2+} concentrations of 150 and 1,000 nM and is unreliable beyond the latter concentration as fura 2 saturates.

For each experiment, the nuclear envelope was again initially Ca\textsuperscript{2+} loaded in incubation solution (extraneur [Ca\textsuperscript{2+}] < 5 nM) (also see above). In all instances, a large, but transient, increase of [Ca\textsuperscript{2+}]\textsubscript{np}, was noted, confirming a [Ca\textsuperscript{2+}]\textsubscript{inr}-to-[Ca\textsuperscript{2+}]\textsubscript{np} gradient (data not shown). Such Ca\textsuperscript{2+}-loaded nuclei were then exposed to IP\textsubscript{3} (10 µM) or cADPr (10 µM) in modified incubation solution in the presence or absence of a highly specific cADPr antagonist, 8-amino-cADPr (18 µM). A rapid sustained elevation of [Ca\textsuperscript{2+}]\textsubscript{np} in fura-dextran loaded nuclei suggested nucleoplasmic Ca\textsuperscript{2+} influx, respectively, through IP\textsubscript{3}Rs and RyRs (Fig. 6). Notably, the response to cADPr was completely abolished with 8-amino-cADPr pretreatment, suggesting that cADPr specifically interacted with its binding site on the RyR. However, expectedly, the response to IP\textsubscript{3} application remained intact even in the presence of 8-amino-cADPr, again suggesting response specificity.

In contrast to the results by Gerasimenko et al. (11), the observed rather than transient responses were sustained. This could be due to either the absence or inactivation of Ca\textsuperscript{2+} efflux mechanisms in our specific experimental conditions. One such mechanism may involve the activation of the IP\textsubscript{4} receptor (28).
DISCUSSION

We provide biochemical and immunochemical evidence for the existence of the type 1 isoforms of both IP$_3$R and RyR in osteoblast nuclear membranes. We also show that both Ca$^{2+}$ channels allow nucleoplasmic Ca$^{2+}$ influx triggered, respectively, by IP$_3$ and cADPr. Furthermore, Ca$^{2+}$ triggers either channel (33); likewise, our demonstration of Ca$^{2+}$-induced Ca$^{2+}$ influx. Such second messenger gating of nuclear Ca$^{2+}$ influx is not only of general biological interest but is also directly relevant to the physiology of the bone-forming osteoblast. Several groups have identified IP$_3$ receptors on a variety of eukaryotic cell nuclear membranes. Nonetheless, evidence for the presence of RyRs on cellular nuclear membranes is somewhat more tenuous, and in this respect, our study provides further biochemical and functional documentation. In fact, RyRs comprise a family of related isoforms, namely types 1, 2, and 3 (34). Of these, the type 1 isoform is expressed almost exclusively in skeletal muscle wherein it couples electrically to the dihydropyridine receptor and gates depolarization-induced Ca$^{2+}$ release (34). It is therefore surprising that RyR-1, rather than the more ubiquitous RyR-2 or RyR-3, is the isoform we identify in osteoblastic cells. It is also notable also that although the presence of IP$_3$Rs on the nuclear membrane was also documented by Guihard et al. (15), the group did not detect any RyR isoforms, including, RyR-1 (15).

A key issue thus arises, What is the precise function of these receptors in nuclear Ca$^{2+}$ homeostasis in general, and in the osteoblast, in particular? Indeed, despite vigorous debate, the dogma of how nuclear Ca$^{2+}$ is regulated still remains unsettled (27, 40). For example, it is unclear whether and, if so, under what circumstances, does the nuclear pore complex become permeable to Ca$^{2+}$ (38). Most electrophysiological studies in isolated nuclei appear to oppose the concept of unrestricted cytosol-to-nucleoplasm Ca$^{2+}$ exchange through the nuclear pore (26, 29). However, if the nuclear pore does become permeable to Ca$^{2+}$, nuclear membrane IP$_3$Rs and RyRs could still conceivably regulate [Ca$^{2+}$]$_{np}$ in response to elevations in cytosolic IP$_3$ and Ca$^{2+}$ In the osteoblast, the latter events will arise from hormone and growth factor receptor activation (17), integrin-matrix interactions (33), and Ca$^{2+}$ transfer through gap junctions (8).

Not much is known about the precise topology of the IP$_3$Rs and RyRs in the nuclear membrane. Humbert et al. (21) have demonstrated quite elegantly that IP$_3$Rs are located in the inner nuclear membrane. The move-
ment of Ca\(^{2+}\) from the perinuclear space to the nucleoplasm also indicates that the large NH\(_2\)-terminus-containing portion of the molecule is nucleoplasmic. Furthermore, we show that IP\(_3\)R and RyR immunoreactivity are preserved when intact nuclei are treated with trypsin; this also indicates an intranuclear location of the two molecules.

Thus, in the presence of a nucleoplasmic IP\(_3\)-binding site, for example, the proposed regulation of nuclear Ca\(^{2+}\) by cytosolic IP\(_3\) must assume that IP\(_3\) diffuses freely through the nuclear pore to activate an inner nuclear membrane IP\(_3\)R. This is by no means certain. In Xenopus laevis oocytes, intracytoplasmic IP\(_3\) injection triggers intranuclear Ca\(^{2+}\) elevation, suggesting a cytosol-to-nucleoplasmic movement of IP\(_3\) (18). Our demonstration of nuclear Ca\(^{2+}\) gating by extranuclear IP\(_3\) and cADPr provides additional evidence for the inner nuclear membrane permeation of second messengers. Gerasimenko et al. (11) have also used extranuclear second messenger to activate nucleoplasmic Ca\(^{2+}\) release in isolated nuclei. In starfish oocytes, however, the cytosolic photolysis of “caged” IP\(_3\) does not trigger a nuclear Ca\(^{2+}\) signal, and intranuclearly injected cADPr exhibits only a limited outward diffusion. Both findings suggest that the second messenger permeability of the nuclear membrane may indeed be restricted (41).

In the absence of second messenger permeation into the nucleus, cytosolic IP\(_3\) must first release Ca\(^{2+}\) into the cytosol from the nuclear envelope. A consequent reduction in [Ca\(^{2+}\)]\(_{\text{cyt}}\), might then, through a positive feedback, trigger Ca\(^{2+}\) influx across the inner nuclear membrane. This is unlikely, as, thus far, IP\(_3\)Rs have been documented not to exist on the outer nuclear membrane (21). It thus becomes challenging, on the basis of presumed topology of IP\(_3\)R and RyR) and the questionable permeability of the inner nuclear membrane to IP\(_3\), to ascribe a role for cytosolic IP\(_3\) in the regulation of nuclear Ca\(^{2+}\). In fact, the generation of both IP\(_3\) and cADPr is possible within the nucleus (2). Namely, phospholipase C\(_{\beta}\) and C\(_{\alpha}\), PI transfer protein, and PI-4- and PI-5-kinases are all present within defined nuclear subcompartments (5, 7, 36). We have recently demonstrated that CD38, a cADPr-producing ribosyl cyclase, is also located in the inner nuclear membrane, with its catalytic site facing nucleoplasmically (1). Nevertheless, their functional significance remains unclear, as do the cellular signals that might conceivably trigger nuclear PI kinase activation.

Despite the paucity of mechanistic information, there is general agreement that nucleoplasmic Ca\(^{2+}\) controls at least two critical nuclear processes, gene transcription and apoptosis (28, 41). Ca\(^{2+}\) can regulate gene expression either directly, through Ca\(^{2+}\)-response elements, or indirectly, via protein kinase C (39, 16). High nuclear Ca\(^{2+}\) enhances the expression of several early response genes, including c-fos, c-jun, jun B, fos B, nur-77, and zif-268, as well as certain late genes, such as the interleukin-2, -3, and -6 genes (12, 14, 15, 19, 39). Notably, both c-fos and interleukin-6 genes are critical to osteoblastic bone formation (15, 30, 31) and should, in principle, be regulated by changes in nuclear Ca\(^{2+}\).

Nevertheless, a direct relationship between nuclear Ca\(^{2+}\) influx and the regulation of osteoblastic genes remains speculative.

Finally, enhanced osteoblast apoptosis is thought to underlie the bone loss associated with senescence (22). Cellular senescence, at least in vitro, is paralleled by an increased RyR expression (20). Furthermore, Ca\(^{2+}\) permits apoptosis by activating certain nuclear proteases, endonucleases, and members of the bcl-2 gene family (23, 32). Thus potential age-related increases in RyR, by enhancing nuclear Ca\(^{2+}\) gating, might result in an elevated [Ca\(^{2+}\)]\(_{\text{nuc}}\) and hence reduced osteoblast survival. Careful mechanistic studies into the permissive effects of Ca\(^{2+}\) on osteoblast apoptosis are therefore urgently warranted.

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