Presence of a Putative Vesicular Inositol 1,4,5-Trisphosphate-Sensitive Nucleoplasmic Ca^{2+} Store†

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ABSTRACT: The inositol 1,4,5-trisphosphate receptors (IP_3Rs) are widely localized in both the heterochromatin and euchromatin regions. We found recently the presence of nucleoplasmic complexes that are composed of phospholipids, IP_3R/Ca^{2+} channels, and Ca^{2+} storage protein chromogranin B (CGB). Close examination and 3D image reconstruction of these complexes revealed numerous vesicular structures with an average diameter of ∼50 nm that are primarily interspersed between the heterochromatins. IP_3 rapidly released Ca^{2+} from these structures, but other inositol phosphates, inositol 1,4-bisphosphate, inositol 1,3,4-trisphosphate, and inositol 1,3,4,5-tetrakisphosphate, failed to release Ca^{2+}. Addition of heparin or IP_3R antibody blocked the IP_3-induced Ca^{2+} releases, indicating the release of Ca^{2+} through the IP_3R/Ca^{2+} channels. Given the presence of the IP_3R/Ca^{2+} channels and Ca^{2+} storage protein CGB in these vesicular structures, we postulate that these vesicles are the IP_3-sensitive nucleoplasmic Ca^{2+} stores. Abundance of the vesicular Ca^{2+} stores between the heterochromatins appeared to imply critical roles these vesicular Ca^{2+} stores play in controlling the Ca^{2+} concentrations of the chromosomes.

Despite the importance of calcium ions in controlling nuclear functions including chromosome replication and transcription control, virtually very little or no information is available regarding the Ca^{2+} control mechanisms in the nucleus. Chromosomes contain 20−32 mM Ca^{2+}, which was shown to fluctuate depending on the replication state; decondensed chromosomes were shown to contain smaller amounts of Ca^{2+}, whereas condensed chromosomes contained larger amounts (*1*). This indicated that the nucleus not only stores a large amount of Ca^{2+} but also has a high capacity Ca^{2+}-buffering ability.

Moreover, the IP_3-mediated nuclear Ca^{2+} release has been known to be essential in the fusion of nuclear vesicles during cell division (*2*). Blocking of Ca^{2+} releases through the IP_3R[1]/Ca^{2+} channels also inhibited the fusion of nuclear vesicles (*2*), underscoring the importance of the IP_3-induced Ca^{2+} releases in nuclear processes. However, the nuclear Ca^{2+} releases have in the past been attributed to the IP_3-induced Ca^{2+} release from the nuclear envelope (NE) through the IP_3R/Ca^{2+} channels that exist in the NE (*3*−*6*). Further, some of these NE membranes have been reported to penetrate into the nucleoplasm, appearing as thin channel-like (*7*) or reticulum-like (*8*) structures, and to contain the IP_3R/Ca^{2+} channels (*8*, *9*). Hence, the IP_3-induced Ca^{2+} release from the nucleoplasmic reticulum was proposed to be directly responsible for the IP_3-induced Ca^{2+} mobilization in the nucleoplasm (*8*, *9*). Nevertheless, it is difficult to reconcile how IP_3 produced at the plasma membrane as a result of agonist application causes a robust release of Ca^{2+} from the nucleoplasmic reticulum deep in the nucleoplasm. Even in the case where IP_3 induced release of Ca^{2+} from the nucleoplasmic reticulum, the question of whether this calcium is the source of the robust Ca^{2+} increases in the nucleoplasm still remains because the nucleoplasmic reticulum occupies a very limited area of the total nucleoplasm (*7*−*9*).

The idea of the IP_3-induced Ca^{2+} release from the limited NE to increase the Ca^{2+} concentrations of the whole nucleus severalfold appears practically impossible considering that the nucleus is a large spherical structure with a diameter of ∼5−6 μm (in the case of bovine chromaffin cells) that is surrounded by a ∼3−5 nm thick membrane. Even in the case where the IP_3R/Ca^{2+} channels of the nucleoplasmic reticulum (*8*, *9*) open in response to IP_3 in the nucleoplasm, it will nevertheless be difficult to explain how the apparently rare and spatially limited nucleoplasmic reticulum Ca^{2+} stores can release sufficiently large amounts of Ca^{2+} to increase the nucleoplasmic Ca^{2+} concentration severalfold. The highly limited presence of NE extensions in the nucleoplasm appears incompatible with the widespread simultaneous increases of Ca^{2+} concentration in the nucleoplasm. It is therefore more likely that the IP_3-induced nuclear Ca^{2+} increases are due to the IP_3-mediated releases through opening of the IP_3R/Ca^{2+} channels located widely inside the nucleus.

Clearly differing from the proposed IP_3-induced Ca^{2+} release from the NE or nucleoplasmic reticulum, the possibility of the presence and operation of the IP_3-mediated nuclear Ca^{2+} control mechanism in the nucleoplasm proper has been implied from the findings that the nucleoplasm contains substantial amounts of all three types of the IP_3Rs (IP_3R-1, -2, and -3) (*10*, *11*), in addition to phosphatidylinositol 4,5-bisphosphate (PIP_2) and phospholipase C (PLC)

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[1] Abbreviations: CGB, chromogranin B; IP_3R, inositol 1,4,5-trisphosphate receptor.

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activity (*12*−*15*). Further, the presence of the high capacity, low affinity Ca^{2+} storage protein chromogranin B (CGB) in the nucleus (*16*) underscored this possibility. We have shown recently that the nucleus contains nucleoplasmic structures that consist of IP_3R/Ca^{2+} channels, Ca^{2+} storage protein chromogranin B, and phospholipids, with a molecular size of $\sim 2-3 \times 10^7$ Da (*17*). We have therefore explored here the possibility of existence of IP_3-sensitive Ca^{2+} stores in the nucleoplasm.

Moreover, we have also investigated the IP_3-mediated Ca^{2+} release properties of this putative vesicular nucleoplasmic Ca^{2+} store and found that it releases Ca^{2+} rapidly in response to IP_3. However, the IP_3-mediated Ca^{2+} release was inhibited by heparin or IP_3R antibody. Other inositol phosphates such as inositol 1,4-bisphosphate, inositol 1,3,4-trisphosphate, and inositol 1,3,4,5-tetrakisphosphate failed to induce Ca^{2+} release from this store, indicating the inositol 1,4,5-trisphosphate-specific nature of the Ca^{2+} store.

EXPERIMENTAL PROCEDURES

Antibodies. Monoclonal anti-mouse CGB antibody (L1BF2) was raised against intact native bovine CGB, and this antibody recognizes a domain within residues 526−575. IP_3R peptides specific to terminal 10−13 amino acids of type 1 (HPPHMNVNPQQPA), type 2 (SNTPHENHHMPPA), and type 3 (FVDVQNCMSR) were synthesized with a carboxy-terminal cysteine, and anti-rabbit polyclonal antibodies were raised. The polyclonal anti-rabbit antibodies were affinity purified on each immobilized peptide, and the specificity of each antibody was confirmed (*18*, *19*). Monoclonal anti-mouse phosphatidylinositol 4,5-bisphosphate (PIP_2) and calreticulin antibodies were from Echelon Biosciences (Salt Lake City, U.S.A.) and Calbiochem, respectively. Polyclonal antibodies for synaptotagmin I and the nuclear protein histone H1 were from Sigma and Upstate Biotechnology, respectively, and the monoclonal antibody for syntaxin 1A was from Serotec (Oxford, U.K.). Polyclonal calnexin antibody was from Calbiochem.

Preparation of Cytoplasmic Proteins and Nucleoplasm of Bovine Chromaffin Cells. The nucleoplasm of chromaffin cells was obtained from the purified nuclei of bovine adrenal chromaffin cells. For this purpose, bovine adrenal medulla (40 g) was cut out from bovine adrenal glands and 5 mL/g medulla of buffer 1 (0.3 M sucrose, 15 mM Tris-HCl, pH 7.5, 0.1 M NaCl) was added. Following mincing and homogenization with a blender, the homogenates were filtered through three layers of cheesecloth and centrifuged for 15 min at 1,000 × *g*. The pellet was then resuspended in 100 mL of buffer 2 (0.25 M sucrose, 10 mM Tris-HCl, pH 7.5, 10 mM NaCl, 3 mM $MgCl_2$, 1 mM DTT, 0.5 mM PMSF) and centrifuged for 10 min at 1,000 × *g*. The supernatant was used as the source of the cytoplasmic proteins, but the pellet that contained the nuclei was resuspended in 60 mL of buffer 2 for further processing. The resuspended pellet was homogenized with a Teflon pestle, and 3 mL of the homogenates was layered over 28 mL of sucrose gradient (1.4−2.2 M) for centrifugation at 98,000 × *g* for 30 min. The nuclei-containing layer was collected, homogenized, and layered on a 2.0 M sucrose solution for centrifugation at 98,000 × *g* for 30 min. The resulting crude nuclear pellet was resuspended again in buffer 2, homogenized, and layered over a 1.8 M sucrose solution for further centrifugation. At this stage, the pellet consisted mostly of the nuclei. To further separate the residual cell debris from the nuclei, the nuclear pellet in buffer 2 was centrifuged at 1,500 × *g* for 20 min. After resuspending the nuclei in buffer 3 (15 mM Tris-HCl, pH 7.5), the purified nuclei were then subjected to sonication, followed by centrifugation at 21,000 × *g* for 30 min. Highly pure nucleoplasm was obtained in the supernatant of this run. All procedures were carried out at 4 °C. To prepare the nucleoplasm for electron microscopy and Ca^{2+} release study, the purified nucleoplasm was concentrated to 2.5−3.0 mg protein/mL and kept frozen at −70 °C for at least 1 h. This freezing step caused the nucleoplasm to aggregate so that it could be pelleted by centrifugation at 21,000 × *g* for 3 min for subsequent use in electron microscopy and Ca^{2+} release measurements.

Purification of the Vesicular Nucleoplasmic Structures. The extracted nucleoplasm (sample 1) from bovine chromaffin cells was first fractionated by Sephacryl S-1000 gel filtration chromatography as described previously (*17*). The fractions containing CGB and the IP_3Rs were pooled and concentrated (sample 2). The concentrated nucleoplasmic sample 2 was further fractionated by sucrose gradient centrifugation. For this 7 mg of the nucleoplasmic proteins in 3 mL of buffer 3 (15 mM Tris-HCl, pH 7.5) was loaded on 28 mL of sucrose gradient solution (0.3−1.5 M sucrose in buffer 3) and centrifuged at 112,000 × *g* for 6 h at 2 °C. Approximately 1.1 mL per fraction was collected, and every other fraction was analyzed by SDS−PAGE and immunoblots. Fractions 5−13 that contained the IP_3Rs and CGB were pooled and used as sample 3 after concentration.

Immunogold Electron Microscopy. For the immunogold electron microscopic study of chromaffin cells and the purified nucleoplasm, the tissue samples from bovine adrenal medulla as well as the pelleted nucleoplasm samples were prepared on Formvar/carbon-coated nickel grids as described previously (*10*). After etching and washing, the grids were placed on 50 μL droplets of solution A (phosphate buffered saline solution, pH 8.2, containing 4% normal goat serum, 1% BSA, 0.1% Tween 20, 0.1% sodium azide) for 30 min. Grids were then incubated for 2 h at room temperature in a humidified chamber on 50 μL droplets of monoclonal anti-mouse CGB or anti-mouse PIP_2, antibody appropriately diluted in solution B (solution A but with 1% normal goat serum), followed by rinses in solution B. The grids were reacted with the 10-nm gold-conjugated goat anti-mouse IgG or IgM, diluted in solution A. For each IP_3R isoform-specific double immunogold labeling experiment, the grids that had gone through the CGB-labeling step with 10-nm particles were reacted once more with each IP_3R isoform-specific antibody labeled with 15-nm gold particles. Controls for the specificity of CGB- and each IP_3R isoform-specific immunogold labeling included (1) omitting the primary antibody, (2) replacing the primary antibody with the preimmune serum, and (3) adding the primary antibody in the excess presence of either purified CGB or each IP_3R isoform-specific peptide that had been used to raise the antibody. After washes in PBS and deionized water, the grids were stained with uranyl acetate (7 min) and lead citrate (2 min) and were viewed with a Zeiss EM912 electron microscope.

High-Voltage Electron Microscopy and 3D Reconstruction. For electron tomography, the purified nucleoplasm samples from bovine adrenal chromaffin cells were sectioned in 250 nm thickness and prepared as described (*10*). The sections placed on the Formvar-coated copper grid were stained with 2% aqueous uranyl acetate and lead citrate, and gold particles were placed on the surface of the sections to provide fiducial points for subsequent image alignment. After carbon coating to enhance their stability in the electron beam, sections on grid were placed in a tilting stage and viewed with a JEM ARM 1300S high-voltage electron microscope (JEOL, Japan, Tokyo) operating at 1250 kV. Once a suitable nucleoplasmic region was selected, the sample was tilted from +60° to −60° with 1° increments, and 121 tilt images at 60,000× magnification were recorded on film. Negatives acquired every 1° tilting interval were scanned, and the digitized images were aligned by using the gold particles in each tilt view as fiducial markers. The tomographic reconstruction from the tilt series was interpreted and modeled using the IMOD program package (*20*). Virtual slices (1.2 nm) were extracted from the tomogram, and the boundaries of several vesicles that were visible in each tomographic slice were traced as contours overlaid on the image. Object surfaces were rendered using the IMOD softwares.

Measurements of Ca^{2+} Release from the Nucleoplasm by Fluorescence Microscopy. To prepare the nucleoplasm for Ca^{2+} release experiments, the freeze−thawed nucleoplasm (2.5−3.0 mg protein/mL buffer 3) was pelleted by centrifugation at 21,000 × *g* for 3 min. The nucleoplasmic pellet was suspended in 1 mL of buffer 3 containing 10 μM EGTA and centrifuged again (wash 1). This washing step was repeated two more times, and the pellet after the third wash was resuspended in buffer 3 at a final concentration of 1.5 mg protein/mL. Then the Ca^{2+} concentration of this nucleoplasmic solution was adjusted with EGTA to ∼0.1 μM and used for Ca^{2+} measurement. To 200 μL (1.5 mg protein/mL) of the nucleoplasmic solution was added fura-2 at a final concentration of 20 μM, and the solution was incubated for 10 min at room temperature. The fura-2-containing sample chamber was then placed on the stage of a Carl Zeiss Axiovert S 100 microscope (Jena, Germany). The nucleoplasmic Ca^{2+} release was analyzed by dual excitation of fura-2 at 340 and 380 nm with a LAMBDA LS xenon arc lamp and LAMBDA 10-2 optical filter changer (Sutter Instrument Co., Novato, U.S.A.). The emission fluorescence signals at 510 nm were collected using a band-pass filter of D510/40 nm (Chroma Technology Corp., Rockingham, U.S.A.) and Hamamatsu C4742-95 digital CCD camera (Hamamatsu-city, Japan). The ratio images were acquired every second and continued with successive addition of inositol phosphates and other test molecules. The changes of fluorescence ratio at the two excitation wavelengths were calculated by MetaFluor software (Universal Imaging Corporation, West Chester, U.S.A.). The influence of sample volume increase in chamber and of chemical dissolving reagent on the fluorescence ratio was tested by adding distilled H_2O and dimethyl sulfoxide.

Optical Emission Spectrometry. The Ca^{2+} content of purified nuclei of chromaffin cells was measured by optical emission spectrometry on a Perkin-Elmer Optima 4300 DV optical emission spectrometer. Dilution was carried out with concentrated nitric acid, and Orion calcium standard solution was used for calibration.

RESULTS

Immunogold Labeling of CGB and IP_3R Isoforms in the Nucleoplasm. In view of the evidence that indicated the presence of IP_3R-CGB-phospholipid complex in the nucleoplasm (*17*), localization of the IP_3Rs and CGB in the nucleoplasm was determined by immunogold electron microscopy using monoclonal CGB and polyclonal IP_3R antibodies (Figure 1A). As shown in Figure 1A, the majority of IP_3R-1-labeling gold (15 nm) particles found in the nucleoplasm were localized next to the CGB-labeling gold (10 nm) particles, demonstrating co-localization of IP_3R-1 and CGB. Likewise, the IP_3R-2 (15 nm)- and CGB (10 nm)-labeling gold particles were also co-localized in the nucleoplasm (Figure 1B), exhibiting a localization pattern similar to that of CGB and IP_3R-1 (Figure 1A). Moreover, this co-localization pattern of the IP_3R isoforms and CGB in the nucleoplasm was also extended to IP_3R-3 (Figure 1C).

Specificity of the monoclonal CGB antibody is shown in Figure 2A and B. Further, to obtain the vesicular nucleoplasmic structures that contain CGB and the IP_3Rs and to investigate the IP_3-dependent Ca^{2+} mobilization function of these structures, we isolated the nucleoplasm of bovine adrenal chromaffin cells, and its purity was determined using antibodies for the nucleoplasmic marker histone H1 and cytoplasmic markers syntaxin 1A, synaptotagmin I (Figure 2C), and ER markers calnexin and calreticulin (cf. Figure 4B). The nucleoplasmic extracts that contained histone H1, were devoid of syntaxin 1A, synaptotagmin I, and calreticulin, but contained a very small amount of calnexin (cf. Figure 4B). The ER marker calnexin was contained in the initial nucleoplasmic sample, but it was removed in the later purification steps and was absent in the purified nucleoplasmic sample (sample 3). On the other hand, the cytoplasmic proteins were devoid of histone H1 but contained all of the cytoplasmic marker proteins. With the exception of a slight calnexin signal in the nucleoplasm, these results show the purity of the nucleoplasmic extracts.

IP_3-Induced Ca^{2+} Release from the Putative Nucleoplasmic Ca^{2+} Store. In view of the co-localization of the IP_3Rs and Ca^{2+} storage protein chromogranin B in the vesicles of the nucleoplasm, the possibility that these vesicles might serve as IP_3-sensitive Ca^{2+} stores arose. Therefore, to test whether IP_3 can release Ca^{2+} from these structures, we added IP_3 to the isolated nucleoplasm and measured the amount of Ca^{2+} released (Figure 3). As shown in Figure 3A, 1 μM IP_3 triggered release of large amounts of Ca^{2+} from the nucleoplasm. Further addition of IP_3 kept on releasing Ca^{2+} until the IP_3 concentration reached ∼10 μM, at which concentration ∼85% of the Ca^{2+} that can be released by 10 μM ionophore was released. The half-maximal Ca^{2+} releasing effect occurred at ∼0.8 μM IP_3 (Figure 3B), which was well within the physiological range and was similar to the K_{app} values shown in other IP_3-mediated Ca^{2+} release studies (*21*).

Despite the robust IP_3-triggered release of Ca^{2+} from the nucleoplasm, no other inositol phosphates such as inositol 1,4-bisphosphate, inositol 1,3,4-trisphosphate, and inositol 1,3,4,5-tetrakisphosphate appeared to mimic the specific Ca^{2+}-releasing property of inositol 1,4,5-trisphosphate. As

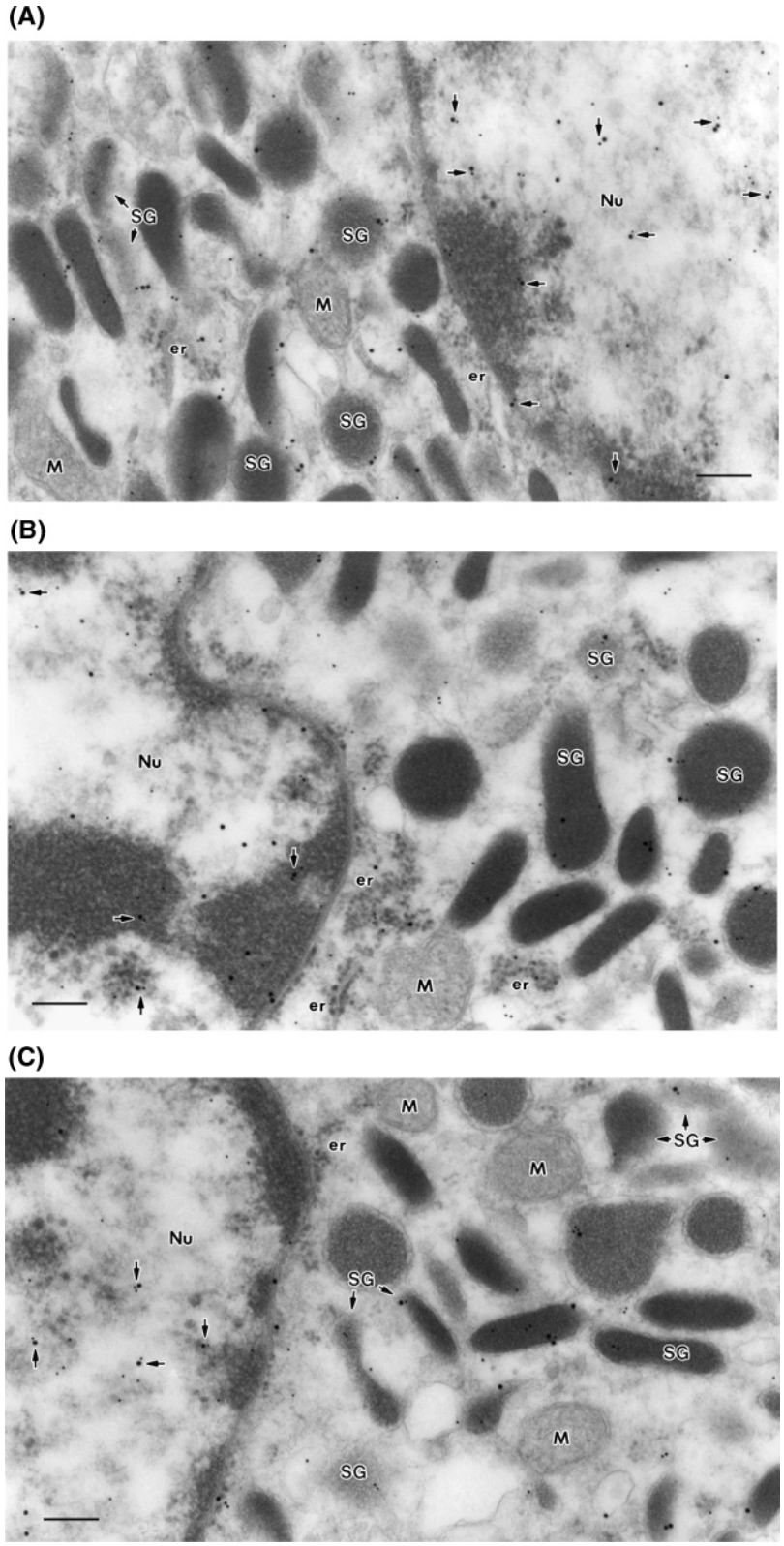

FIGURE 1: Localization of CGB and IP_3R in the nucleoplasm. (A) Chromaffin cells were double immunolabeled for CGB (10 nm gold) and the IP_3R-1 (15 nm gold) with monoclonal CGB antibody and the affinity purified type 1-specific IP_3R antibody. The CGB- and IP_3R1-labeling gold particles are localized in secretory granules (SG), the endoplasmic reticulum (er), and nucleus (Nu) but not in mitochondria (M). The areas where CGB (10 nm)- and IP_3R1 (15 nm)-labeling gold particles are localized next to each other are marked with arrows. Identical experiments were carried out with IP_3R antibodies specific for type 2 (B) and 3 (C). Bar = 200 nm.

shown in Figure 3C and D, inositol 1,4-bisphosphate and inositol 1,3,4,5-tetrakisphosphate failed to release Ca^{2+} from the nucleoplasm, nor was inositol 1,3,4-trisphosphate able to release Ca^{2+} from the nucleoplasm though the nucleoplasm was perfectly capable of releasing Ca^{2+} in response to inositol 1,4,5-trisphosphate. Nevertheless, IP_3 failed to release Ca^{2+} from the nucleoplasm in the presence of IP_3R antibody (Figure 3E), and this inhibitory effect of IP_3R antibody on the IP_3-dependent Ca^{2+} release was not mimicked by preimmune serum or purified IgG.

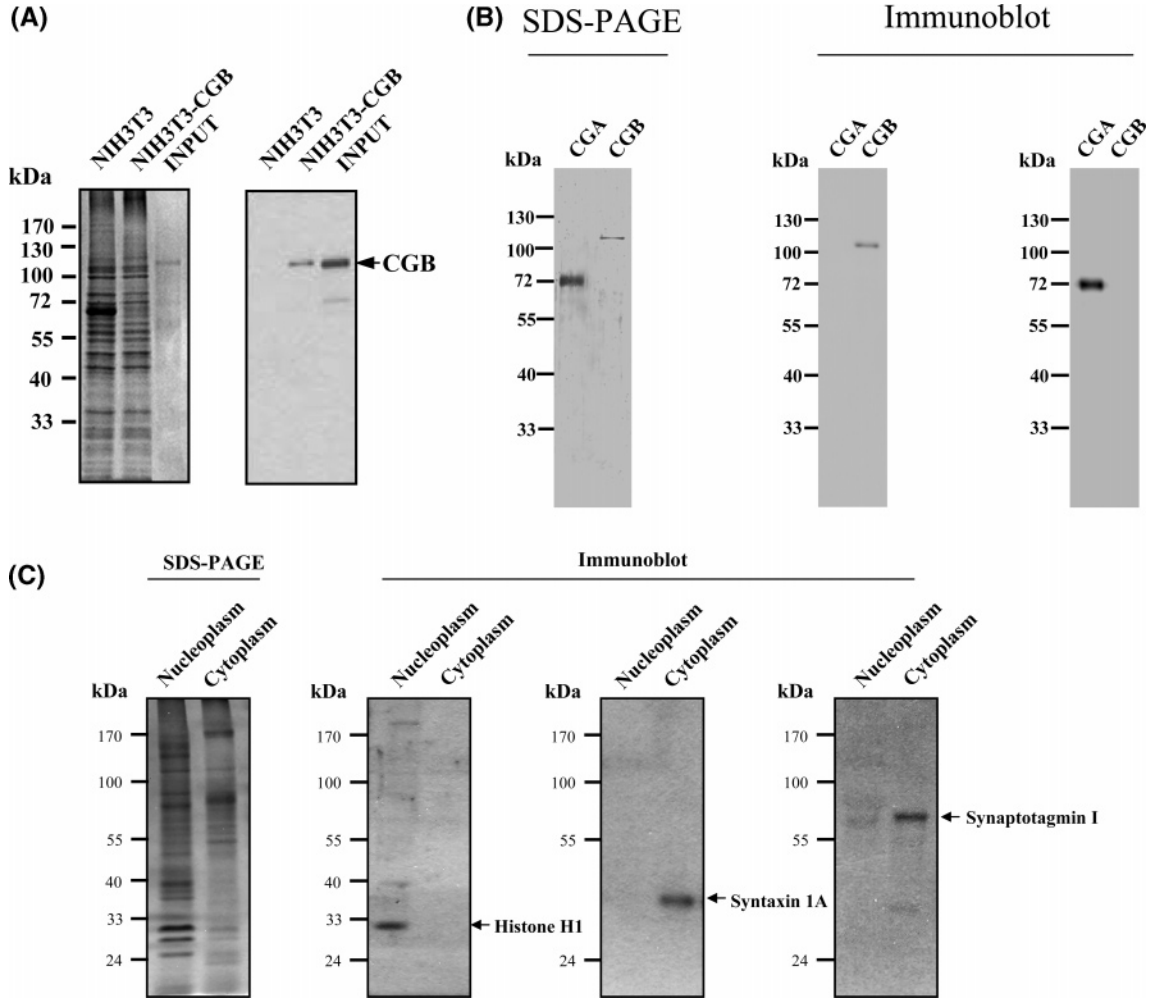

FIGURE 2: Specificity of antibodies and isolation of the nucleoplasm. (A) Cell extracts from normal and CGB-transfected NIH3T3 cells (*16*) were separated on a PAGE gel (10 μg/lane) along with 0.2 μg of purified CGB (input) (left panel). The same proteins were immunoblotted with monoclonal CGB antibody (right panel). (B) Purified bovine CGA (0.5 μg) and CGB (0.2 μg) were separated on a PAGE gel (left) and were also immunoblotted with the same monoclonal CGB (middle) and polyclonal CGA (right) antibodies. (C) Separation of nucleoplasmic and cytoplasmic proteins (10 μg/lane) and immunoblot analysis using antibodies for nucleoplasmic protein histone H1 (10 μg/lane) and cytoplasmic proteins syntaxin 1A (10 μg/lane) and synaptotagmin I (20 μg/lane).

Further, the IP_3R/Ca^{2+} channel antagonist heparin also blocked the IP_3-induced Ca^{2+} release (Figure 3F), whereas neither Ca^{2+} ATPase inhibitor thapsigargin nor antimycin A or oligomycin exerted any effect on the IP_3-dependent Ca^{2+} release from these structures, demonstrating the specificity of the inositol 1,4,5-trisphosphate-dependent Ca^{2+} release property of the nucleoplasmic Ca^{2+} store. These results not only indicate that the IP_3-mediated Ca^{2+} release is through the IP_3R/Ca^{2+} channel but also show that the vesicular nucleoplasmic structures contain large amounts of Ca^{2+} that can be released in response to IP_3, underscoring the presence of large amounts of stored Ca^{2+} in the nucleus.

Purification of the Vesicular Nucleoplasmic Ca^{2+} Store. To purify the IP_3-sensitive vesicular nucleoplasmic structures, the nucleoplasm (sample 1) that showed IP_3-dependent Ca^{2+} releases was fractionated on a Sephacryl S-1000 gel filtration column (1.5 cm $\times$ 58 cm), and the fractions that contained the IP_3Rs and CGB were pooled (sample 2). These fractions were concentrated and subjected to sucrose gradient centrifugation for further purification (Figure 4A). The nucleoplasmic CGB and IP_3R were found in the low-density fractions (Figure 4A), which were pooled and concentrated (sample 3) for further characterization. Figure 4B shows not only the purification of the nucleoplasmic samples but also the enrichment of the IP_3R and CGB in the purified samples. The phospholipid content also increased as the nucleoplasmic structures were purified (Figure 4C).

Examination of nucleoplasmic sample 3 by electron microscopy revealed the presence of numerous vesicular structures with an average diameter of ~50 nm (Figure 4D), clearly demonstrating the purification of the vesicular structures. These structures were widespread in the electron-dense chromatin regions, and they often appeared to exist in clusters. The IP_3-induced Ca^{2+} releases from the purified nucleoplasmic structures were significantly greater than those of unpurified sample (Figure 4E). The amount of Ca^{2+} released from sample 3 in response to 4 μM IP_3 was ~0.6 μM, whereas that from samples 1 and 2 was 0.1 and 0.3 μM, respectively (Figure 4E). In light of the fact that present nucleoplasmic samples had a protein concentration of 2 mg/mL and these samples were used in 0.2 mL volumes, 0.6 μM Ca^{2+} released translates into 0.3 nmol Ca^{2+} released/mg proteins of the nucleoplasmic sample.

The EC_{50} concentrations of IP_3 did not appear to differ between the nucleoplasmic contents from the three different

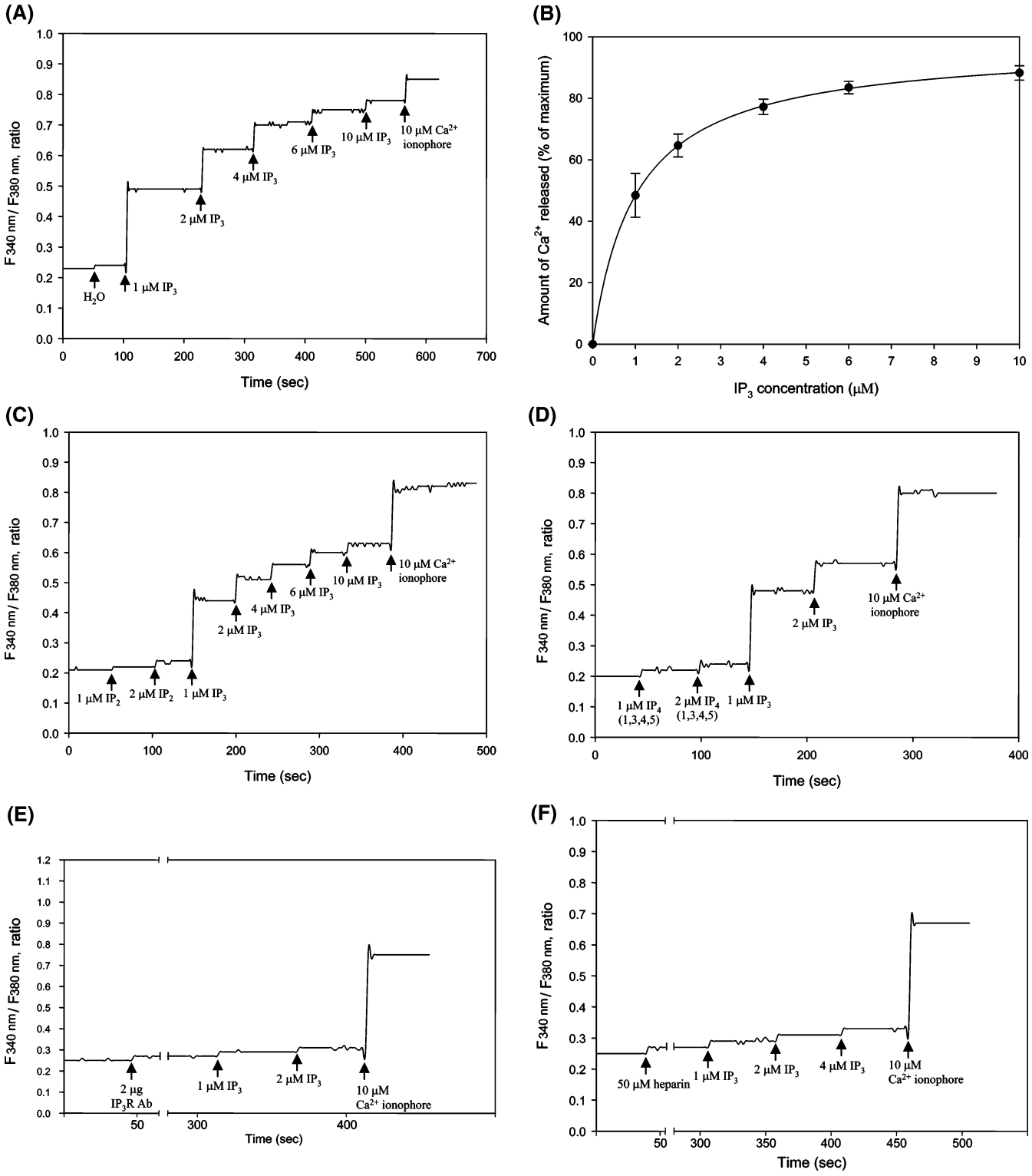

FIGURE 3: Ca^{2+} release from the isolated nucleoplasm. (A) The Ca^{2+} releases were recorded after a series of incremental additions of 1−4 μM inositol 1,4,5-trisphosphate to the nucleoplasmic solution (200 μL, 1.5 μg protein/μL) containing 20 μM fura-2. Each indicated concentration of IP_3 represents the amount of cumulative IP_3 added, and in the end a cumulative total of 10 μM IP_3 in 5 μL was added. The data shown are representative of similar results repeated 15 times. (B) The amount of Ca^{2+} released as a result of IP_3 addition is expressed as percentage of maximum releasable Ca^{2+}. The fluorescence changes caused by 10 μM ionophore was set as the maximum change, and the IP_3-induced fluorescence changes as a function of added IP_3 concentration are shown (mean ± SD, $n = 15$). (C) Additions of 1 μM inositol 1,4-bisphosphate (IP_2) to the same nucleoplasmic solution, followed by inositol 1,4,5-trisphosphate. (D) Additions of 1 μM inositol 1,3,4,5-tetrakisphosphate (IP_4), followed by inositol 1,4,5-trisphosphate. (E) Two microliters (1 μg/μL) of affinity purified IP_3R-1 antibody was added to the nucleoplasmic solution (200 μL) and the Ca^{2+} release was measured. (F) The IP_3R/Ca^{2+} channel antagonist heparin was added at a final concentration of 50 μM, followed by the addition of inositol 1,4,5-trisphosphate. The data shown in C−F are representative of similar results repeated 7−10 times.

steps, but the total amount of Ca^{2+} released by the same concentration of IP_3 differed markedly between the three groups, clearly indicating gradual purification of the IP_3-sensitive nucleoplasmic Ca^{2+} store.

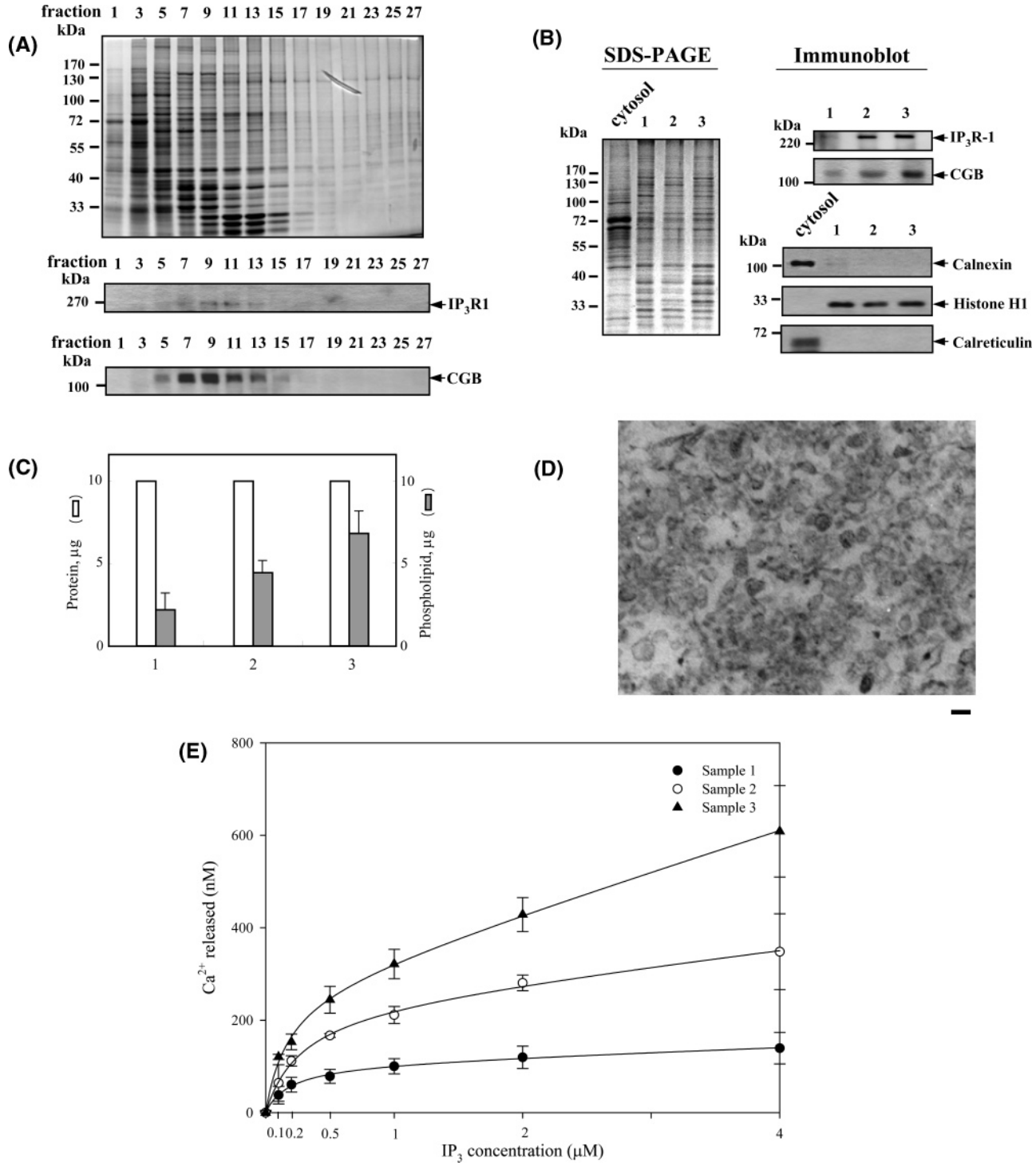

FIGURE 4: Purification of the vesicular structures. **(**A) Seven milligrams of nucleoplasmic proteins in 3 mL of 15 mM Tris-HCl, pH 7.5 were separated on 28 mL of sucrose gradient (0.3−1.5 M), and 40 μL aliquots from each fraction (1.1 mL/fraction) were separated on a 10% SDS-polyacrylamide gel (top panel). Identical SDS−polyacrylamide gels were analyzed by immunoblot analysis using antibodies for CGB and IP_3R-1 (bottom panel). (B) The nucleoplasmic contents from each step of the purification were separated on a 10% SDS−polyacrylamide gel (10 μg/lane) (left). The same proteins were analyzed by immunoblot analysis using antibodies for the IP_3R-1, CGB, nucleoplasmic protein histone H1, and the ER marker proteins calnexin and calreticulin. Numbers 1, 2, and 3 stand for the sample numbers, 1 being the starting nucleoplasm, 2 the sample after the S-1000 chromatography, and 3 the purified sample after the sucrose gradient centrifugation. (C) Phospholipid contents in the nucleoplasmic samples as determined by the method described (*17*). (D) Electron micrograph of purified nucleoplasm, sample 3. Note that numerous vesicular structures are visible. Bar = 50 nm. (E) The amounts of Ca^{2+} released were expressed as a function of IP_3 added to samples 1−3.

Presence of Phosphatidylinositol 4,5-Bisphosphate. Considering the presence of large amounts of phospholipids in the CGB or IP_3R immunoprecipitates of the nucleoplasmic contents (*17*), the presence of phospholipids in the purified spherical structures was examined by immunogold electron microscopy using the PIP_2-specific anti body (Figure 5A). The PIP_2- and IP_3R-labeling gold particles (15 nm) localized primarily to the membranous regions of the spherical structures with an average diameter of ~50 nm, indicating the presence of phospholipid membranes

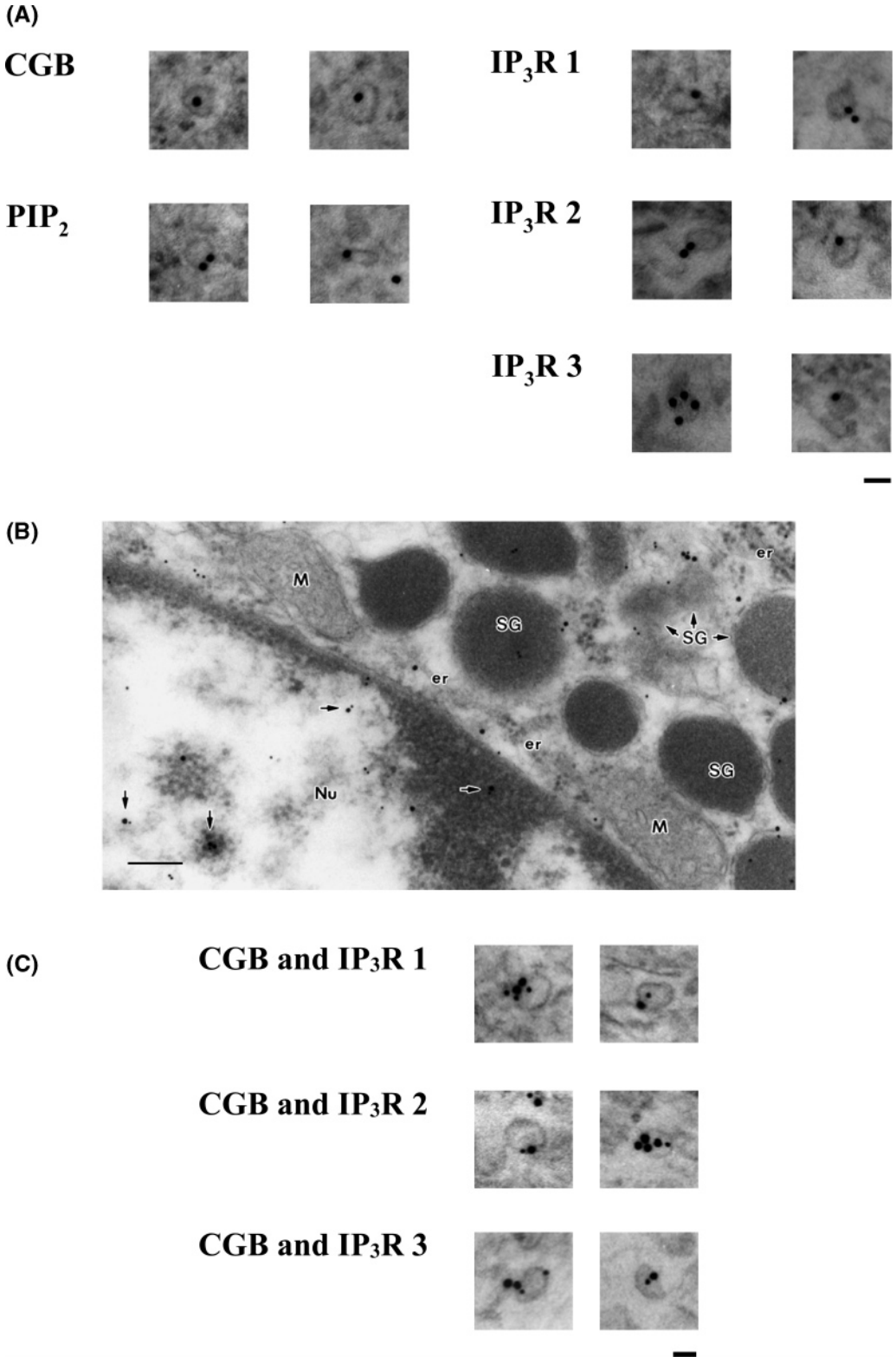

FIGURE 5: Immunogold electron microscopy. (A) Purified sample 3 was examined by immunogold electron microscopy using antibodies for CGB, PIP_2, and each IP_3R isoform (15 nm gold). The PIP_2- and IP_3R-labeling gold particles localized primarily in the membrane region of the vesicles. Bar = 50 nm. (B) Chromaffin cells were double immunolabeled for CGB (10 nm gold) and PIP_2 (15 nm gold) with polyclonal anti-rabbit CGB antibody and monoclonal anti-mouse PIP_2 antibody. The CGB- and PIP_2-labeling gold particles are localized in secretory granules (SG), the endoplasmic reticulum (er), and the nucleus (Nu). The areas where CGB (10 nm)- and PIP_2 (15 nm)-labeling gold particles are localized next to each other in the nucleus are marked with arrows. Bar = 200 nm. (C) The purified sample 3 was also double immunolabeled with each IP_3R isoform (15 nm) and CGB (10 nm) antibodies. Bar = 50 nm.

in these nucleoplasmic structures. The CGB-labeling gold particles also localized in the vesicular structures (Figure 5A) as reported before (*17*).

Most of the PIP_2-labeling gold particles localized in the nucleoplasm were present next to the CGB-labeling gold particles (Figure 5B), thus indicating the coexistence of the

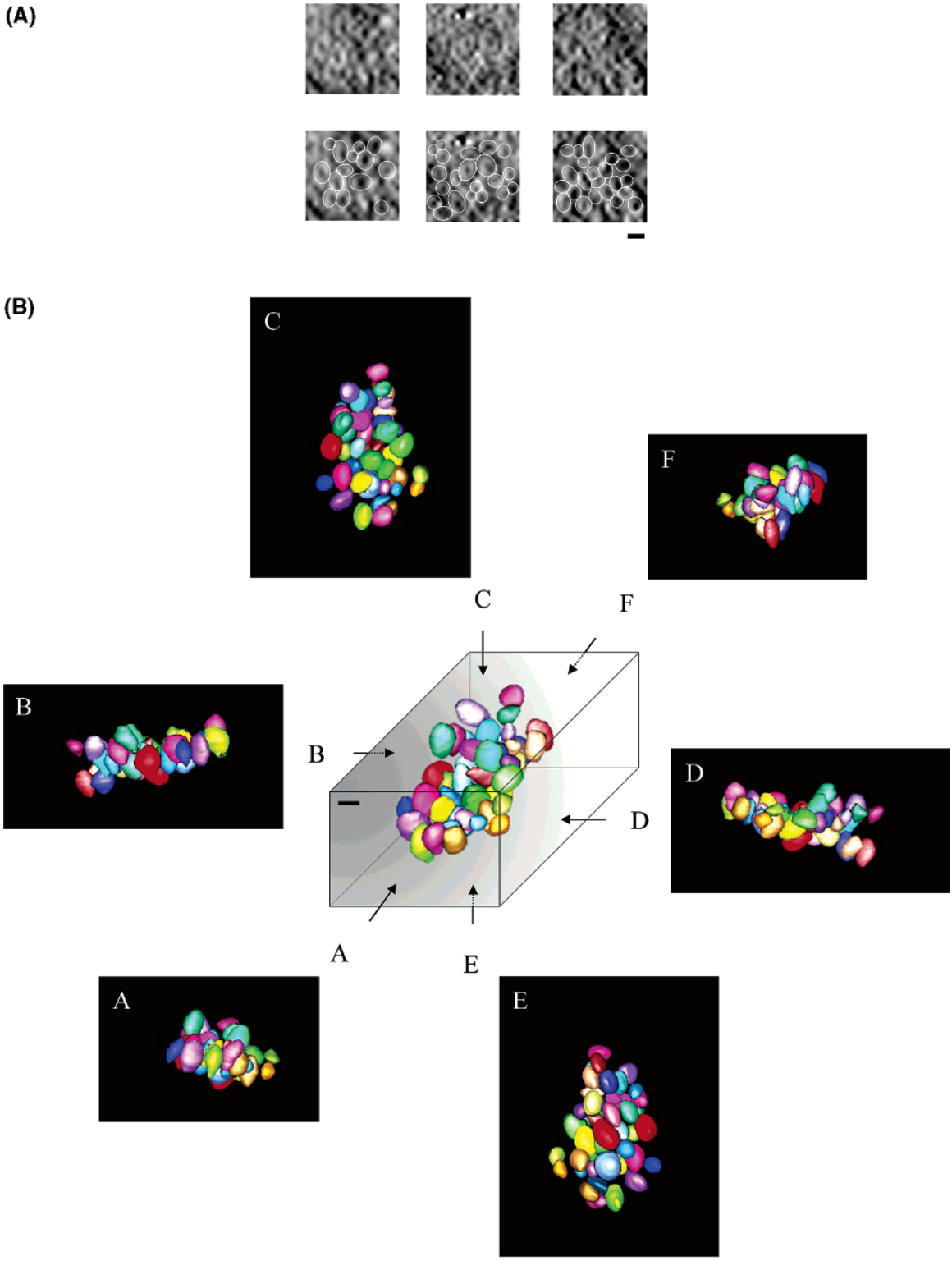

FIGURE 6: 3D image construction. (A) Purified nucleoplasmic sample 3 was subjected to electron tomography, and 1.2-nm tomographic slices were obtained from a 250-nm thick sample. Three of the tomographic slices were shown along with the interpretive drawings (bottom). Bar = 50 nm. (B) 3D images of 44 vesicles from a randomly chosen area of sample 3 were reconstructed using the IMOD program (*20*) from the vesicle images shown on the serial tomographic slices. 3D images of the vesicles are shown in their original locations and sizes in the nucleoplasm, albeit not in exact scale, along with the views from six different angles. Bar = 50 nm.

phospholipid and CGB. Moreover, CGB was also shown to colocalize with the IP_3Rs in the spherical structures (Figure 5C). These results strongly suggest that the spherical structures are vesicles that contain the IP_3R/Ca^{2+} channels and CGB. Although the vesicular structures were not big enough to accommodate multiple gold particles, they contained occasionally more than one CGB- or IP_3R-labeling gold particle.

High-Voltage Electron Microscopy and 3D Image Construction. In view of the presence of the vesicular structures in the nucleoplasm, we next subjected the purified nucleoplasmic vesicular structures of bovine adrenal chromaffin

cells to high-voltage electron microscopy to obtain the 3D image of these vesicles by electron tomography. The purified nucleoplasm was hence sectioned in 250-nm thickness, these sections were examined using a high-voltage electron microscope at 1250 kV, and tomograms were obtained. Serial 1.2-nm slices were extracted from a tomogram, and three of these are shown in Figure 6A, each showing a number of vesicular images. The vesicular structures appeared to exist in clusters, which were estimated to be well above tens of thousands in the nucleus, but each vesicle showed a clear boundary with a relatively clear inner lumen. Hence, we traced the vesicular structures of the tomographic slices in order to reconstruct the 3D images. Figure 6B shows the 3D images of 44 vesicular structures in a randomly chosen area of the nucleoplasm, and movies of these images rotating in space are available in Supporting Information. These vesicles did not have a uniform shape but appeared as eclipsed vesicles with some variations in sizes, ranging ~40−60 nm in diameter with an average diameter of ~50 nm. The diameter of ~50 nm suggested that these vesicles are a bit larger than ribosomes and could contain several lumenal as well as membrane proteins, along with large amounts of lumenal calcium.

Determination of the Total Amount of Calcium in the Nucleus. Since the IP_3-induced Ca^{2+} release from the nucleoplasmic Ca^{2+} stores indicated the presence of large amounts of Ca^{2+} in the nucleus, it was of interest to determine the total amount of calcium in the nucleus. Hence, we determined the total calcium content of the purified nuclei of bovine adrenal chromaffin cells using an optical emission spectrometer (Perkin-Elmer Optima 4300 DV) and found that the nuclei contain 11.4 ± 3.0 (mean $\pm$ SD, $n = 4$) mM Ca^{2+}. This is the second largest amount of calcium found in subcellular organelles in chromaffin cells next to secretory granules, which contain 40 mM Ca^{2+} (*22*, *23*). Since secretory granules are supposed to contain such a large amount of calcium and function as an IP_3-sensitive Ca^{2+} store (*24*−*27*) due to the presence of chromogranins A and B (*28*), it is also highly likely that the vesicular nucleoplasmic structures, which contain chromogranin B and the IP_3R/Ca^{2+} channels, store highly concentrated nuclear calcium and function as an IP_3-sensitive Ca^{2+} store.

DISCUSSION

Present results demonstrate not only the existence of putative vesicular IP_3-sensitive nucleoplasmic Ca^{2+} stores but also the 3D structures of these stores that are composed of phospholipids, IP_3R/Ca^{2+} channels, and chromogranin B. The presence of chromogranin B, which binds ~90 mol Ca^{2+}/mol with a K_d of 1.5 mM (*19*), and the IP_3R/Ca^{2+} channels in the nucleus suggest that this vesicular structure is the IP_3-sensitive nucleoplasmic Ca^{2+} store. In particular, the finding that ~80 μM chromogranin B is present in the nucleus of adrenal chromaffin cells (*29*) appears to be of direct relevance. From the nuclear concentration and the high capacity Ca^{2+}-binding property, chromogranin B is expected to bind a millimolar range of Ca^{2+} in the nucleus, an amount sufficient to control a wide range of nuclear Ca^{2+} concentrations.

Existence of the IP_3R/Ca^{2+} channels in the nucleus has long been considered to be limited to the nuclear envelope (NE) (*3*−*6*) or the extension of the NE deep in the nucleoplasm (*7*−*9*) until we showed in recent studies that large amounts of IP_3R isoforms (types 1, 2, and 3) exist in the nucleoplasm proper as well (*10*, *11*). In view of the fact that the IP_3Rs are present in the ER membranes, the presence of the IP_3Rs in the NE or the extension of the NE was considered a natural consequence. However, the presence of the IP_3Rs in the nucleoplasm proper came as a surprise given that the IP_3Rs are integral membrane proteins, and membrane structures, with the exception of the NE extensions (*7*−*9*), are not known to exist in the nucleoplasm. Despite the demonstrated presence of all three IP_3R isoforms in the nucleoplasm (*11*), it was still not clear where the calcium that can be released through the IP_3R/Ca^{2+} channels is stored in the nucleoplasm. In this regard, present results that demonstrate the presence of numerous vesicular nucleoplasmic Ca^{2+} stores suggest that the millimolar nuclear Ca^{2+} can be stored in these vesicles and these IP_3-sensitive nucleoplasmic Ca^{2+} stores could mediate the fusion of nuclear vesicles.

Therefore, along with the fact that CGB is a vesicular Ca^{2+} storage protein, it is reasonable to assume that these vesicular structures are the IP_3-sensitive nucleoplasmic Ca^{2+} stores that store large amounts of calcium that can be released in response to inositol 1,4,5-trisphosphate. The inability of other inositol phosphates such as inositol 1,4-bisphosphate, inositol 1,3,4-trisphosphate, and inositol 1,3,4,5-tetrakisphosphate to induce Ca^{2+} release indicates that this nucleoplasmic IP_3-sensitive Ca^{2+} store releases Ca^{2+} through the IP_3R/Ca^{2+} channels.

Moreover, from the fact that the IP_3R/Ca^{2+} channels interact with chromogranin B in both the ER and secretory granules (*30*), the nucleoplasmic vesicles appear to meet the minimum requirements for an IP_3-sensitive Ca^{2+} store that can release stored calcium in response to IP_3. In this context, there would be little reason for the nucleus to depend on the cytoplasmic Ca^{2+} for its immediate Ca^{2+} needs. In other words, the immediate Ca^{2+} needs in the nucleus such as the fusion of nuclear vesicles, chromosome replication, and various gene transcriptions could be met within the nucleus without relying on the Ca^{2+} that may come in from the cytoplasm. Likewise, there would be little reason to pump out the nuclear Ca^{2+} through the nuclear pore complex; rather, the nuclear Ca^{2+} may be sequestered by this nucleoplasmic Ca^{2+} store through the IP_3R/Ca^{2+} channels.

Although the 3D images of only 44 vesicles were shown in Figure 6B, a large number of these vesicles were present in the nucleoplasm and were localized primarily in clusters near the heterochromatin regions. Given the abundance of the vesicles observed in the electron micrographs, it appeared that their numbers in the nucleus could be well above tens of thousands. Of particular note is that the vesicles appeared to be interspersed between the electron-dense heterochromatin regions, and this unique distribution pattern may reflect the potential physiological roles these Ca^{2+} stores play in the nucleus.

The Ca^{2+} concentration for a diploid set of chromosomes was shown to exist in the 20−32 mM range, which roughly translates into binding of one Ca^{2+} ion per every 20−30 nucleotides (*1*). Measurement of the bound and unbound cations by secondary ion mass spectrometry of cryofractured interphase and mitotic cells revealed a cell-cycle-dependent

fluctuation in Ca^{2+} concentrations. Ca^{2+}, Mg^{2+}, Na^{+}, and K^{+} were identified as essential participants in the maintenance of chromosome structure, particularly at mitosis due to their functions in DNA electrostatic neutralization and chromosome condensation (*1*). In this respect, the abundant presence of vesicular Ca^{2+} stores between the heterochromatins would also be ideal to maintain the structural integrity of the chromosomes. Decondensed chromosomes were shown to contain smaller amounts of Ca^{2+}, whereas condensed chromosomes contained larger amounts (*1*). In this context, the millimolar fluctuation in nuclear Ca^{2+} concentrations in the nucleus demands a high capacity, low affinity Ca^{2+} buffering capacity in the nucleus, and this would not be possible unless there exist high capacity, low affinity Ca^{2+}-binding proteins in the nucleoplasm. In the case of cells that do not contain CGB, it is likely that functionally equivalent proteins could serve similar roles.

It is tempting to think that the fluctuation of the Ca^{2+} concentrations in the chromosomes could be possible via the release and sequestration of Ca^{2+} by these small, yet numerous vesicular Ca^{2+} stores that are positioned near the chromosomes. Release and sequestration of Ca^{2+} at multiple locations along the chromosomes would enable these small vesicular Ca^{2+} stores to effectively control the amount of Ca^{2+} available for the chromosomes. In this case, the small size as well as the abundance of these Ca^{2+} stores along the chromatins will be critical in meeting the Ca^{2+} needs of the chromosomes. Since transcriptional activities and chromosome replication can occur in many locations simultaneously, the localized Ca^{2+} needs in numerous locations could also be met without difficulty.

Further, given that the nucleus is estimated to contain ~11 mM Ca^{2+}, it is likely that these nucleoplasmic vesicles store a major share of nuclear Ca^{2+} in addition to the storage in chromosomes. Yet by virtue of the IP_3R/Ca^{2+} channels these stored Ca^{2+} could be in a dynamic equilibrium with the rest of the nucleus and be released in a moment's notice upon the introduction of IP_3, a product that can be produced through an inositide signaling system. Indeed, the nucleus has been reported to contain a phosphoinositide signaling system of its own, including the molecules such as phosphatidylinositol 4,5-bisphosphate, phospholipase C, IP_3, and diacylglycerol (DAG) (*12*−*15*). Existence of these molecules and the phosphoinositide signaling system implied in nucleo production of signaling molecules IP_3 and DAG, which suggested the presence and operation of signaling systems involving these molecules in the nucleus. Evidence in support of the operation of an inositide signaling system in the nucleus also includes the observation that the IP_3-induced Ca^{2+} release in the nucleus stimulated the fusion of nuclear vesicles in NE assembly, whereas the fusion of nuclear vesicles was inhibited when the Ca^{2+} releases through the IP_3R/Ca^{2+} channels was blocked (*2*).

The functional coupling between chromogranin B and the IP_3Rs has been shown to play a crucial role in enabling the ER to function as an IP_3-sensitive Ca^{2+} store (*30*). Moreover, chromogranins and the IP_3Rs were shown to localize in specific subcellular locations where the highest intracellular Ca^{2+} releases occur in both the epidermal growth factor-differentiated PC12 cells and hippocampal neurons (*31*), highlighting the critical roles the IP_3Rs and CGB play in controlling the intracellular Ca^{2+} concentrations. In this regard, there will be little reason for the nucleus to depend on the cytoplasmic Ca^{2+} for its immediate Ca^{2+} needs in nuclear activities.

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SUPPORTING INFORMATION AVAILABLE

Movies of the 3D images of 44 vesicles from a randomly chosen area of sample 3 rotating in space. This material is available free of charge via the Internet at http://pubs.acs.org.

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