Presence of a Nucleoplasmic Complex Composed of the Inositol 1,4,5-Trisphosphate Receptor/Ca^{2+} Channel, Chromogranin B, and Phospholipids[†]

Seung Hyun Yoo,* Seong Won Nam, Seong Kwon Huh, Seon Young Park, and Yang Hoon Huh

National Creative Research Initiative Center for Secretory Granule Research and Department of Biochemistry, Inha University College of Medicine, Jung Gu, Incheon 400-712, Korea

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ABSTRACT: Although the inositol 1,4,5-trisphosphate (IP_3) induced nuclear Ca^{2+} releases have been shown to play key roles in nuclear functions, the presence and operation of the IP_3-dependent Ca^{2+} control mechanism in the nucleoplasm have not been shown. Recently, we found the presence of a high-capacity, low-affinity Ca^{2+}-storage protein chromogranin B (CGB) and all three IP_3 receptor (IP_3R) isoforms in the nucleoplasm, localizing widely in both the heterochromatin and euchromatin regions. In view of the essential role of CGB−IP_3R coupling in IP_3-dependent Ca^{2+} release in the endoplasmic reticulum, the potential coupling between CGB and the IP_3Rs in the nucleoplasm was investigated. Hence, we found in the present study the presence of a nucleoplasmic complex, which is composed of the IP_3R, CGB, and phospholipids, with an estimated molecular mass of $\sim 2-3 \times 10^7$ Da, suggesting the possibility of the presence of an IP_3-sensitive Ca^{2+} store in the nucleoplasm. Moreover, double-labeling immunogold electron microscope studies showed the colocalization of all three IP_3R isoforms with CGB to the extent that the majority of each IP_3R isoform-labeling gold particles found in the nucleoplasm was literally next to the CGB-labeling gold particles. In line with the potential existence of an IP_3-dependent vesicular nucleoplasmic Ca^{2+} store, our preliminary results indeed showed a sudden release of Ca^{2+} from a putative nucleoplasmic Ca^{2+} store in response specifically to IP_3 but not to inositol 1,4-bisphosphate or inositol 1,3,4,5-tetrakisphosphate.

Despite the critical roles calcium ions play in controlling nuclear functions including chromosome replication and transcription control (*1*), very little information is available regarding the Ca^{2+} control mechanisms in the nucleus. In a recent study, chromosomes were shown to contain 20−32 mM Ca^{2+}, and the chromosomal Ca^{2+} concentration was shown to fluctuate depending on the chromosome replication state (*2*). This indicates that the nucleus not only stores a large amount of Ca^{2+} but also has a high capacity Ca^{2+}-buffering ability.

Underscoring the importance of nuclear Ca^{2+}, IP_3-mediated nuclear Ca^{2+} release has been known to be essential in the fusion of nuclear vesicles during cell division (*3*). However, the nuclear Ca^{2+} releases have often been attributed to the IP_3-induced Ca^{2+} release from the nuclear envelope (NE)[1] through the IP_3R/Ca^{2+} channels that exist in the NE (*4*−*8*). Furthermore, some NE membranes have been shown to penetrate into the nucleoplasm, appearing as thin channel-like (*9*) or reticulum-like (*10*) structures. This nucleoplasmic reticulum was reported to contain the IP_3R/Ca^{2+} channels, enabling the nucleoplasmic reticulum to function as an IP_3-sensitive Ca^{2+} store (*10*). In view of the presence of IP_3Rs in the NE (*4*−*7*), the existence of IP_3Rs in the nucleoplasmic reticulum was not surprising.

Nevertheless, there was still a question of how IP_3 produced as a result of agonist application causes a robust release of Ca^{2+} from the nucleoplasmic reticulum deep in the nucleoplasm. The IP_3 that opens the IP_3R/Ca^{2+} channels of the nucleoplasmic reticulum should also open the NE IP_3R/Ca^{2+} channels ahead of the nucleoplasmic reticulum IP_3R/Ca^{2+} channels because the nucleoplasmic reticulum is the extension of the NE into the nucleoplasm (*9*, *10*). In this regard, it appeared likely that the nucleoplasm contained the IP_3R/Ca^{2+} channels of its own, which are different from those of the NE.

Indeed, we have recently found the presence of all three isoforms of IP_3Rs (IP_3R-1, -2, and -3) in the nucleoplasm of both neuroendocrine and nonneuroendocrine cells (*11*), thus strongly suggesting the presence and operation of an IP_3-dependent Ca^{2+} control mechanism in the nucleus. The IP_3Rs were widely present in both the heterochromatin and euchromatin regions, implying essential roles of the IP_3R/Ca^{2+} channels in the nucleus. The possibility of the presence and operation of an IP_3-mediated nuclear Ca^{2+} control mechanism in the nucleoplasm has also been implied from the findings that the nucleoplasm contains phosphatidylinositol 4,5-bisphosphate and phospholipase C activity (*12*−*16*).

Moreover, the nucleus also contains a high-capacity, low-affinity Ca^{2+}-storage protein, chromogranin B (*17*), which binds ~90 mol of Ca^{2+}/mol with a dissociation constant (K_d) of 1.5 mM (*18*). In view of the need of the nucleus to control 20−32 mM Ca^{2+} (*2*), it will not be possible for the nucleus

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* To whom correspondence should be addressed. Tel: 82-32-890-0936. Fax: 82-32-882-0796. E-mail: shyoo@inha.ac.kr.

[1] Abbreviations: CGB, chromogranin B; IP_3R, inositol 1,4,5-trisphosphate receptor; NE, nuclear envelope; ER, endoplasmic reticulum.

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to control such high concentrations of nuclear Ca^{2+} unless there exists a high-capacity, low-affinity Ca^{2+}-buffering capacity in the nucleus. In this regard, the high-capacity, low-affinity Ca^{2+}-binding property of chromogranin B could serve the need of the nucleus well. This possibility was further strengthened by the fact that CGB couples to the IP_3Rs not only at the intragranular pH 5.5 but also at a near physiological pH 7.5 (*19*), thereby activating the IP_3R/Ca^{2+} channels (*20*). This IP_3R/Ca^{2+} channel-activating role of CGB was proven to be essential in the IP_3-dependent Ca^{2+} release from the endoplasmic reticulum (ER) (*21*).

Coupling of CGB to the IP_3R/Ca^{2+} channels in the ER also underscored the possibility of the same coupling in the nucleus. We have hence investigated the potential coupling of CGB to the IP_3Rs in the nucleus and found the existence of the CGB−IP_3R complex in the nucleoplasm. Moreover, phospholipids were also present in the CGB−IP_3R complex, further suggesting the possibility of the presence of a vesicular IP_3R−CGB structure that could potentially function as an IP_3-sensitive nucleoplasmic Ca^{2+} store. Our preliminary results indeed indicated that IP_3 induces a rapid Ca^{2+} release from a putative nucleoplasmic Ca^{2+} store and that this Ca^{2+}-releasing effect of IP_3 is not mimicked by inositol 1,4-bisphosphate (IP_2) or inositol 1,3,4,5-tetrakisphosphate (IP_4).

EXPERIMENTAL PROCEDURES

Antibodies. IP_3R peptides specific to the 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 following the procedure described (*22*), and the specificity of each antibody was confirmed (*18*). The antibody for the nucleoplasm marker protein histone H1 was obtained from Upstate Biotechnology and calreticulin antibody was from Calbiochem.

Preparation of Cytoplasmic Proteins and Nucleoplasmic Contents of Bovine Chromaffin Cells. The nucleoplasmic contents of chromaffin cells were 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 1000*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 1000*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 98000*g* for 30 min. The nuclei-containing layer was then collected, homogenized, and layered on a 2.0 M sucrose solution for centrifugation at 98000*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 1500*g* for 20 min. After the nuclei were resuspended in buffer 3 (15 mM Tris-HCl, pH 7.5), the purified nuclei were then subjected to sonication, followed by centrifugation at 21000*g* for 30 min. Highly pure nucleoplasmic contents were obtained in the supernatant of this run. All of the procedures were done at 4 °C.

Sucrose Gradient Centrifugation. For fractionation of the nucleoplasmic contents, 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 (1.4−2.2 M sucrose in buffer 3) and centrifuged at 112000*g* for 6 h at 2 °C. By collecting ~1.1 mL per fraction, 28 fractions were collected, and each fraction was analyzed by SDS−PAGE and immunoblots.

Sephacryl S-1000 Chromatography of the Nucleoplasmic Contents. For a gel filtration chromatography of the nucleoplasmic contents, 6 mg of the nucleoplasmic proteins in 3 mL of buffer 3 (15 mM Tris-HCl, pH 7.5) was loaded on a Sephacryl S-1000 column (1.5 × 58 cm) equilibrated with buffer 3 and eluted with buffer 3 using an FPLC system (Pharmacia). Each fraction was analyzed by SDS−PAGE and immunoblots.

Determination of Phospholipids. Quantitative determination of phospholipids present in the nucleoplasmic contents was carried out according to the published method (*23*), which utilized the reaction of molybdenum with phospholipids. For a qualitative determination of phospholipids, appropriate nucleoplasmic contents were dissolved in a chloroform−methanol (1:1) mixture and were analyzed using a thin-layer chromatography as described previously (*24*).

Co-immunoprecipitation of CGB and the IP_3R from the Nucleoplasmic Extracts of Adrenal Chromaffin Cells. To perform co-immunoprecipitation experiments, 200 μg of the nucleoplasmic proteins was mixed first in 500 μL of the pH 7.5 buffer (20 mM Tris-HCl, pH 7.5, 0.1 M KCl, 0.1% Triton X-100). The mixture was precleaned by incubating with protein A−Sepharose for 1 h at 4 °C, followed by centrifugation at 22000*g* for 5 min. For immunoprecipitation experiments the supernatant (~200 μL) was briefly sonicated and incubated with 10 μL (1 μg/μL) of the polyclonal anti-rabbit CGB or type I-specific IP_3R (IP_3R-1) antibody for 1 h at 4 °C. Then 60 μL of a 1:1 slurry of protein A−Sepharose in the pH 7.5 buffer was added to the mixture and incubated for 1 h. The mixtures were then put into a minicolumn (0.7 × 5.5 cm), and the immunoprecipitate was washed five times with the pH 7.5 buffer. The immunoprecipitate was separated from protein A−Sepharose by eluting with 0.2 M glycine, pH 2.2, which was then used for quantitation of its protein and phospholipid. In addition, the proteins in the immunoprecipitate were separated on 7.5% SDS−polyacrylamide gels and subjected to immunoblot analysis using antibodies for IP_3R-1 and CGB.

Immunocytochemical Localization of CGB and IP_3R Isoforms. For the double-labeling immunogold electron microscopic study of chromaffin cells, the tissue samples from bovine adrenal medulla were prepared on Formvar/carbon-coated nickel grids as described (*11*). After etching and washing, the grids were placed on 50 μL droplets of solution A (phosphate saline solution, pH 8.2, containing 4% normal

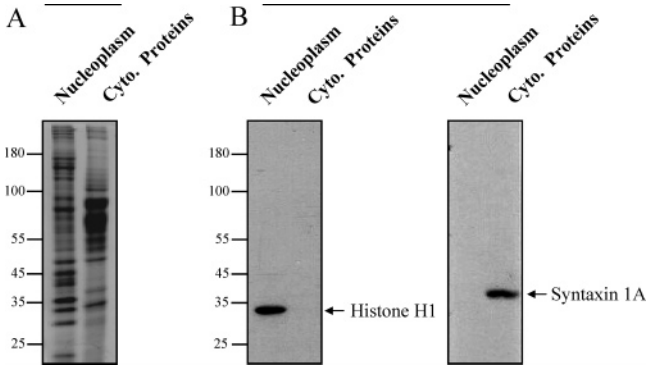

FIGURE 1: SDS−PAGE and immunoblot analysis of the nucleoplasmic proteins of chromaffin cells. (A) Nucleoplasmic and cytoplasmic proteins of bovine adrenal chromaffin cells were separated on a 10% SDS−polyacrylamide gel (10 μg/lane) and visualized by Coomassie blue staining. (B) The same proteins were analyzed by immunoblot analysis using antibodies for nucleoplasmic protein histone H1 and cytoplasmic protein syntaxin 1A.

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 anti-rabbit CGB 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-rabbit IgG 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.

RESULTS

To determine the purity of the nucleoplasmic extracts, the nucleoplasmic extracts were subjected to immunoblot analysis using antibodies for the nucleoplasmic marker histone H1 and cytoplasmic marker syntaxin 1A (Figure 1). As shown in Figure 1, the nucleoplasmic extracts contained histone H1 but were devoid of the cytoplasmic marker protein syntaxin 1A while the cytoplasmic proteins were devoid of histone H1 but contained syntaxin 1A, indicating the purity of the nucleoplasmic extracts.

Cofractionation of CGB and the IP_3R. To determine whether the nuclear CGB and IP_3Rs cofractionate, the nucleoplasmic extracts were subjected to sucrose gradient centrifugation, and the location of CGB and the IP_3R in the fractions was examined using immunoblot analysis. As shown in Figure 2, both CGB and the IP_3R were shown to be present in fractions 3−7, indicating the presence of both CGB and the IP_3R in the same fractions, whereas histone H1 was present in fractions 7−11.

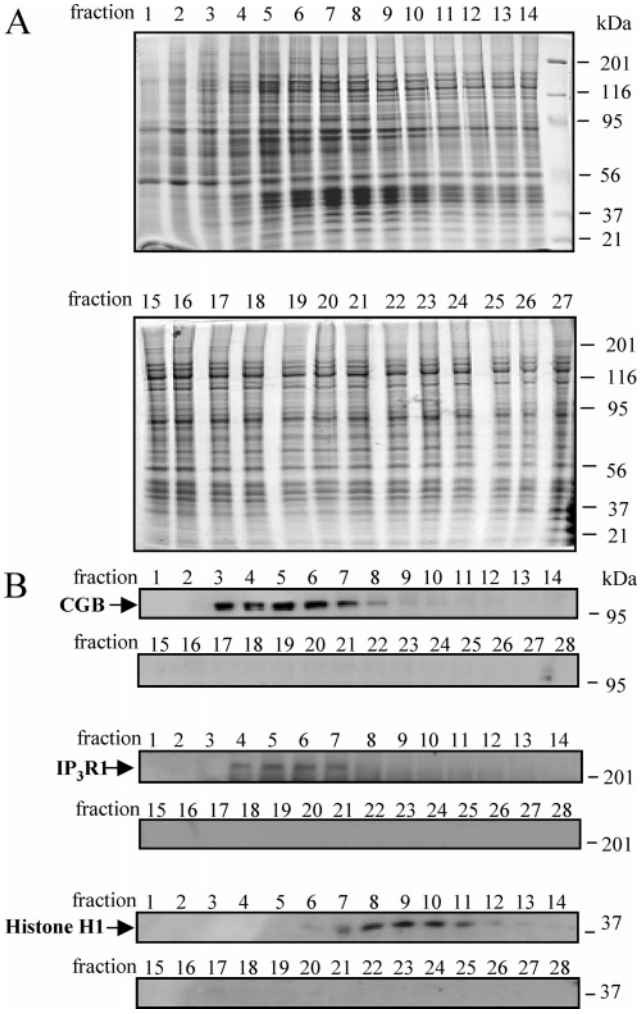

FIGURE 2: Sucrose gradient centrifugation and immunoblot analysis of the nucleoplasmic proteins of chromaffin cells. (A) 7 mg of nucleoplasmic proteins in 3 mL of 15 mM Tris-HCl, pH 7.5, was separated on 28 mL of sucrose gradient (1.4−2.2 M), and 40 μL aliquots from each fraction (1.1 mL/fraction) were separated on a 10% SDS−polyacrylamide gel and visualized by Coomassie blue staining. (B) Identical SDS−polyacrylamide gels were analyzed by immunoblot analysis using antibodies for CGB, IP_3R-1, and histone H1.

The possibility of cofractionation of CGB and the IP_3R was further investigated using a gel filtration chromatography (Figure 3). In the Sephacryl S-1000 column (1.5 × 58 cm) chromatography, CGB was shown to elute in fractions 15−21 (Figure 3B). The IP_3R eluted in fractions 15−21, the same fractions where CGB was eluted. These results indicated the presence of CGB and the IP_3R in the same fractions and confirmed the sucrose gradient centrifugation result (Figure 2). Since the Sephacryl S-1000 column is a gel filtration column with a fractionation range of 5×10^5 to 1×10^8 Da, it was possible to estimate the size of an eluting molecule or particle within this range. The largest nucleoplasmic content eluted at ~50 mL of elution volume, suggesting the upper fractionation limit of the Sephacryl S-1000 column, yet the nuclear CGB and IP_3R eluted at ~70 mL of elution volume (Figure 3D). Hence, from the elution position of CGB and the IP_3R in the Sephacryl S-1000 column, it was estimated that the nuclear CGB and the IP_3R eluted in a position where a globular protein with a molecular mass of $\sim 2-3 \times 10^7$ Da elutes (Figure 3D). Given that the

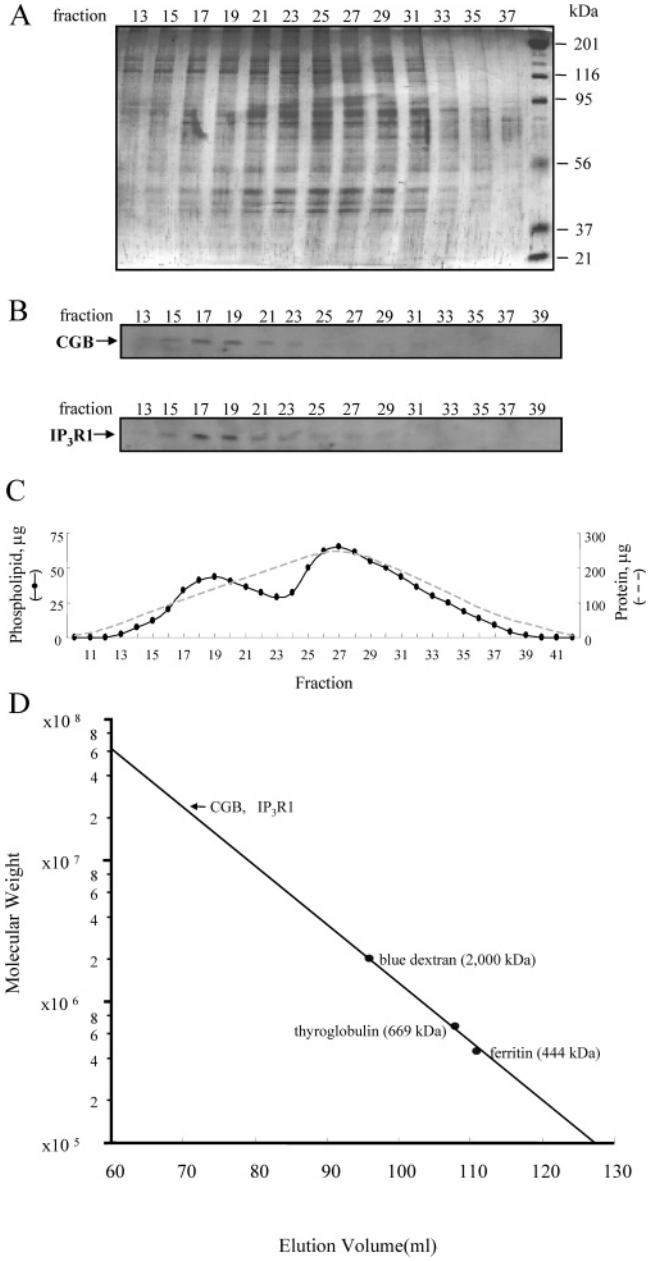

FIGURE 3: Sephacryl S-1000 gel filtration chromatography of the nucleoplasmic extracts and immunoblot and phospholipid analyses. (A) 6 mg of nucleoplasmic proteins in 2 mL of 15 mM Tris-HCl, pH 7.5, was subjected to Sephacryl S-1000 column (1.5 × 58 cm) chromatography, and 42 μL aliquots from each fraction (3 mL/fraction) were separated on a 10% SDS−polyacrylamide gel and visualized by Coomassie blue staining. (B) Identical SDS−polyacrylamide gels were analyzed by immunoblot analysis using antibodies for CGB and IP$_3$R-1 to locate the elution positions of CGB and the IP$_3$R-1. (C) The phospholipid elution profile also indicates a slight enrichment of phospholipids in fractions 15−21. (D) Chromogranin B and the IP$_3$R-1 were eluted in the same position, fractions 15−21, where a globular protein with a molecular mass of ∼2−3 × 10^7 Da elutes.

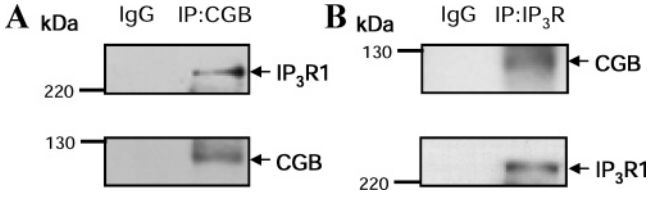

FIGURE 4: Co-immunoprecipitation of CGB and the IP$_3$R. (A) Protein extracts from the nucleoplasmic contents were immunoprecipitated with CGB antibody, and the immunoprecipitates were separated on a 7.5% SDS gel for immunobloting with IP$_3$R-1 antibody (upper panel) and CGB antibody (lower panel), respectively. (B) Protein extracts from the nucleoplasmic contents were immunoprecipitated with IP$_3$R-1 antibody, and the immunoprecipitates were separated on a 7.5% SDS gel for immunobloting with CGB antibody (upper panel) and IP$_3$R-1 antibody (lower panel), respectively. Preimmune IgG was used as control antibody. 260 kDa IP$_3$R-1 was co-immunoprecipitated with 110 kDa CGB.

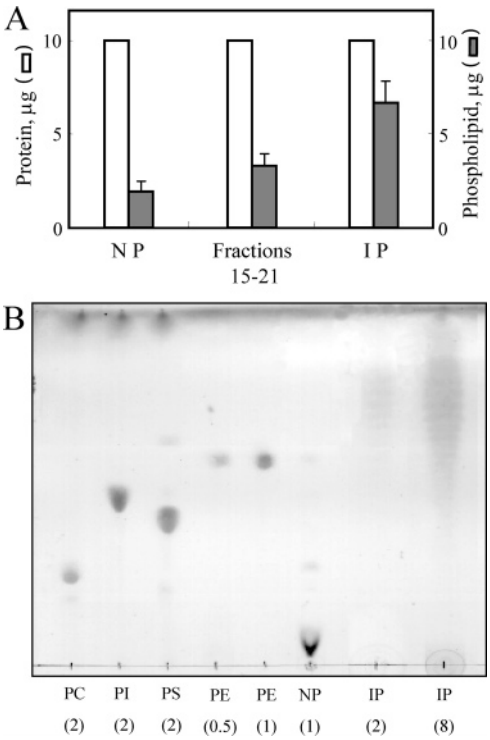

FIGURE 5: Determination of phospholipids in the CGB or IP$_3$R immunoprecipitate. (A) Quantitative determination of phospholipids in the nucleoplasmic extracts (NP), the pooled fractions 15−21 of the Sephacryl S-1000 column chromatography (fractions 15−21), and the CGB immunoprecipitate (IP) was carried out using molybdenum-based reagents as described (*23*), using phosphatidylcholine, phosphatidylinositol, and phoshatidylethanolamine as standards. (B) Qualitative analysis of the phospholipids was done by thin-layer chromatography as described (*24*). 2 μg each of phosphatidylcholine (PC), phosphatidylinositol (PI), and phosphatidylserine (PS) and 0.5 and 1 μg of phosphatidylethanolamine (PE) were used, along with ∼1 μg of phospholipids from the nucleoplasmic extract (NP) and ∼2 and ∼8 μg of phospholipids from the CGB immunoprecipitate (IP). The numbers in parentheses indicate the amount (μg) of phospholipids used. Similar results were also obtained with the IP$_3$R immunoprecipitate.

molecular weight of CGB and the IP$_3$R-1 is 72000 and 260000, respectively, these results suggest that CGB and the IP$_3$R are components of a complex that consisted of several CGB and the IP$_3$R molecules.

To confirm that CGB and the IP$_3$R form a complex in the nucleoplasm, co-immunoprecipitation experiments were also carried out (Figure 4). As shown in Figure 4, immunoprecipitation of the nucleoplasmic extracts with CGB antibody precipitated not only CGB but also the IP$_3$R. Similarly, immunoprecipitation of the nucleoplasmic extracts with IP$_3$R antibody also precipitated both the IP$_3$R and CGB, indicating the presence of the CGB−IP$_3$R complex in the nucleoplasmic extracts.

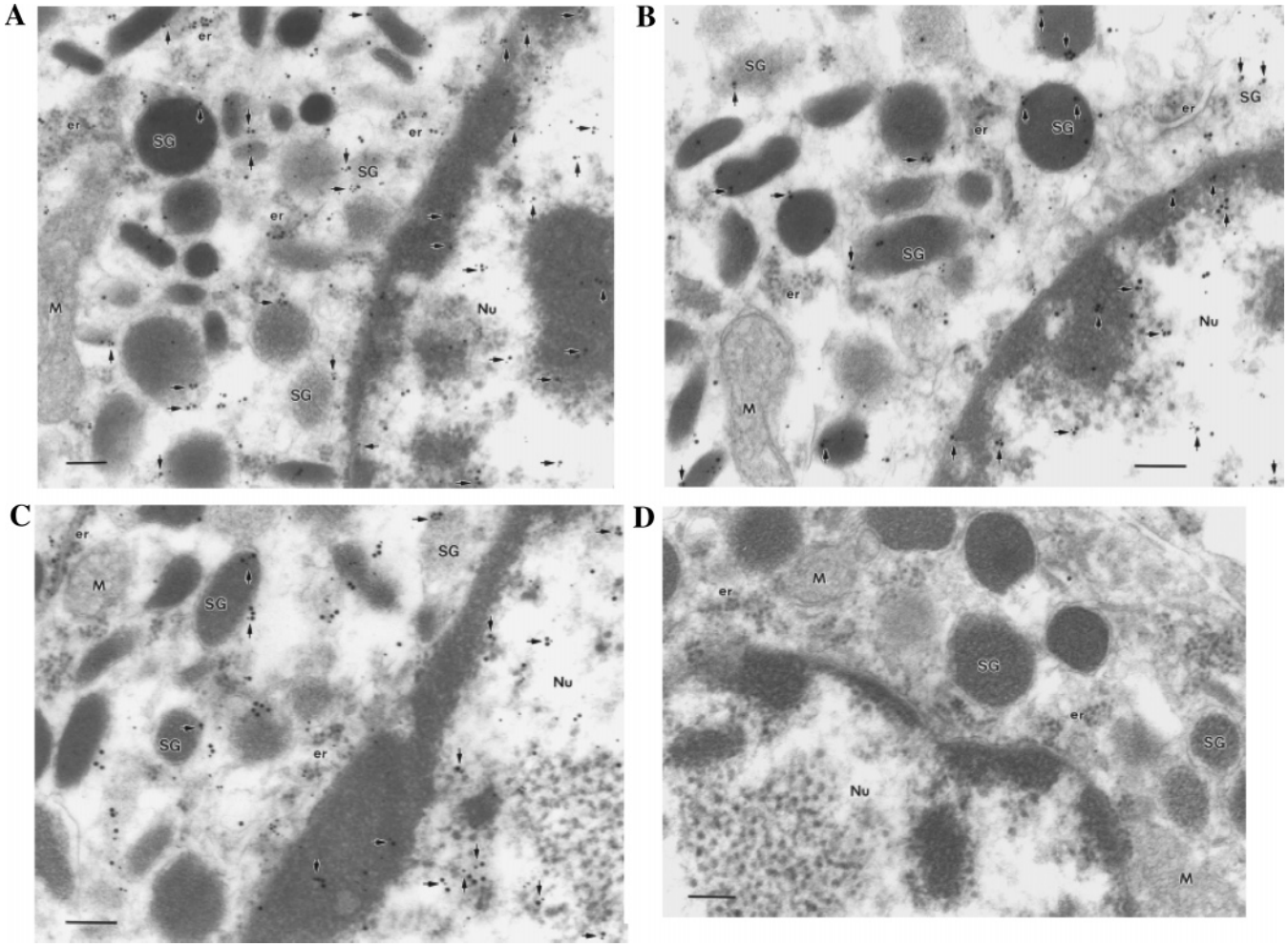

FIGURE 6: Immunogold electron microscopy showing the localization of the IP_3R isoforms and CGB in adrenal chromaffin cells. Bovine adrenal medullary chromaffin cells were double immunolabeled for CGB (10 nm gold) and the IP_3R-1 (A), IP_3R-2 (B), or IP_3R-3 (C) (15 nm gold) with the affinity-purified each isoform-specific IP_3R antibody. Identical experiments were carried out either in the absence of the primary antibody or with the preimmune serum in place of the primary antibody (D). The CGB and IP_3R isoform-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_3R isoform (15 nm) labeling gold particles are localized next to each other are marked with arrows. Bar = 200 nm.

Despite the complex formation between CGB and the IP_3R, it was not clear whether the estimated CGB−IP_3R complex with a size of $\sim 2-3 \times 10^7$ Da consists of proteins only or a combination of proteins and other components such as phospholipids. Since phospholipids are known to be present in the nucleus (*12*−*16*), we have examined the presence of phospholipids in the nucleoplasmic extracts (Figure 5). Quantitation of the phospholipids using standard procedures (*23*) indicated the presence of a substantial amount of phospholipids in the nucleoplasmic extracts. As shown in Figure 5A, the phospholipid weight in the nucleoplasmic extracts was shown to be approximately 20% (w/w) of the protein weight detected in the nucleoplasmic extracts. However, the phospholipid weight in fractions 15−21 of the Sephacryl S-1000 column chromatography (cf. Figure 3B,C) increased to ∼30−35% (w/w) of the protein weight in the same fractions (Figure 5A).

Since this result suggested the possibility that the nucleoplasmic CGB−IP_3R complex also contained phospholipids, the presence of phospholipids in the CGB−IP_3R complex was further investigated. Hence, the pooled fractions of fractions 15−21 of the Sephacryl S-1000 column (cf. Figure 3B,C) were subjected to co-immunoprecipitation using both CGB and IP_3R antibodies, and the presence of phospholipids in the CGB−IP_3R co-immunoprecipitates was examined (Figure 5A). As shown in Figure 5A, the phospholipid weight in the CGB immunoprecipitate further increased to ∼65−70% (w/w) of the protein weight, demonstrating not only the presence of phospholipids in the CGB−IP_3R complex but also the abundance of phospholipids in the complex.

Further, to analyze the phospholipids that exist in the immunoprecipitates by thin-layer chromatography (TLC), the phospholipids were extracted from the CGB immunoprecipitate, and the extracted phospholipids were separated on a TLC plate (*24*) (Figure 5B). As shown in Figure 5B, large amounts of phsopholipids that appeared to migrate at speeds similar to or faster than those for phosphatidylinositol and phosphatidylethanolamine were present in the immunoprecipitates. Although the nucleoplasmic extracts appeared to contain phosphatidylcholine and phosphatidylethanolamine (Figure 5B), it was not apparent what kind of phospholipids contributed to the IP_3R−CGB−phospholipid complex. Since phospholipids are markedly smaller than either CGB or IP_3R, the presence of proteins and phospholipids even in a 3:2 (w/

Table 1: Distribution of the CGB- and IP_3R-1-Labeling Gold Particles in the Nucleoplasm

	IP_3R-1		CGB	
	total	coupled	total	coupled
heterochromatin	27	13	44	17
euchromatin	10	7	21	6

w) ratio indicates the presence of a significantly larger number of phospholipid molecules in the complex than that of either CGB or IP_3R molecules. Therefore, it appears likely that the nucleoplasmic IP_3R−CGB−phospholipid complex is composed of a vesicular phospholipid structure that includes several CGB and the IP_3R molecules.

Double Labeling of CGB and IP_3R Isoforms in the Nucleoplasm. In view of the evidence that indicated the presence of the IP_3R−CGB−phospholipid complex in the nucleoplasm, colocalization of CGB and the IP_3Rs in the nucleoplasm was further examined using double-labeling immunogold electron microscopy. As shown in Figure 6A, the CGB- and IP_3R-1-labeling gold particles were found in the ER, secretory granules, and the nucleus but not in mitochondria, in line with the previous reports that showed the presence of CGB and the IP_3Rs in the nucleus (*11*, *17*). Agreeing with direct coupling between CGB and the IP_3Rs, and probably reflecting the key functional interaction between the two, many of the CGB-labeling gold particles (10 nm) were localized literally next to the IP_3R-1-labeling gold particles (15 nm) in these organelles. In particular, the colocalization of CGB- and IP_3R-1-labeling gold particles in the nucleus was such that more than half of all the IP_3R-1-labeling gold particles present in the nucleus were found next to the CGB-labeling gold particles. Figure 6A shows that out of a total of 27 IP_3R-1-labeling gold particles in the heterochromatin regions 13 IP_3R-1-labeling gold particles are localized next to the CGB-labeling gold particles while out of a total of 10 IP_3R-1-labeling gold particles in the euchromatin regions 7 gold particles are localized next to the CGB-labeling gold particles (Table 1), indicating that 20 out of the total 37 IP_3R-1-labeling gold particles present in the nucleus are localized next to the CGB-labeling gold particles.

The colocalization pattern of CGB and IP_3R-2 was similar to that of CGB and IP_3R-1. As shown in Figure 6B, the IP_3R-2-labeling gold particles were found in the ER, secretory granules, and the nucleus but not in mitochondria. Agreeing with the presence of IP_3R-2 in the nucleus (*11*), the IP_3R-2-labeling gold particles (15 nm) were present in the nucleus. Figure 6B shows that out of a total of 24 IP_3R-2-labeling gold particles in the heterochromatin regions 16 IP_3R-2-labeling gold particles are found next to the CGB-labeling gold particles while out of a total of 6 IP_3R-2-labeling gold particles in the euchromatin regions 1 gold particle is found next to the CGB-labeling gold particles (Table 2), indicating that 17 out of the total 30 IP_3R-2-labeling gold particles present in the nucleus are localized next to the CGB-labeling gold particles.

Likewise, the CGB- and IP_3R-3-labeling gold particles were also localized in the ER, secretory granules, and the nucleus but not in mitochondria (Figure 6C). Consistent with the presence of IP_3R-3 in the nucleus (*11*), the IP_3R-3-labeling gold particles (15 nm) were present in the nucleus.

Table 2: Distribution of the CGB- and IP_3R-2-Labeling Gold Particles in the Nucleoplasm

	IP_3R-2		CGB	
	total	coupled	total	coupled
heterochromatin	24	16	39	12
euchromatin	6	1	6	2

Table 3: Distribution of the CGB- and IP_3R-3-Labeling Gold Particles in the Nucleoplasm

	IP_3R-3		CGB	
	total	coupled	total	coupled
heterochromatin	22	12	26	8
euchromatin	7	4	6	2

Similar to the results of other IP_3R isoforms, Figure 6C shows that 12 IP_3R-3-labeling gold particles out of a total of 22 in the heterochromatin regions and 4 gold particles out of a total of 7 in the euchromatin regions are localized next to the CGB-labeling gold particles (Table 3), indicating that 16 out of 29 IP_3R-3-labeling gold particles present in the nucleus are localized next to the CGB-labeling gold particles. Consistent with the absence of the IP_3Rs in mitochondria, virtually no IP_3R-labeling gold particles were found in mitochondria except the particles shown as a result of nonspecific binding.

To further determine whether there is any relationship between the putative IP_3-sensitive vesicular nucleoplasmic Ca^{2+} store and the previously reported IP_3-sensitive nucleoplasmic reticulum Ca^{2+} store (*10*), the localization of the ER marker protein calreticulin in the nucleus was examined by immunogold electron microscopy. As shown in Figure 7, calreticulin was shown to localize in the ER and NE but was absent in secretory granules and the nucleoplasm. Nevertheless, CGB localized to secretory granules, the ER, and nucleoplasm as expected. Despite the occasional colocalization of calreticulin with CGB in the ER and NE, no calreticulin was shown to localize in the nucleoplasm, clearly distinguishing the vesicular nucleoplasmic complex from the nucleoplasmic reticulum.

DISCUSSION

The present results show the presence of a nucleoplasmic complex that consists of the IP_3Rs, CGB, and phospholipids, which could potentially function as an IP_3-dependent Ca^{2+} store in the nucleus. Chromogranin B is a high-capacity, low-affinity Ca^{2+}-storage protein, binding >90 mol of Ca^{2+}/mol with a dissociation constant of ~1.5 mM (*18*), and is known to activate the IP_3R/Ca^{2+} channels (*20*). Although CGB has long been known as a major secretory granule matrix protein, it is also present in the nucleus at ~40 μM, playing a role in transcription control (*17*). Similarly, the IP_3Rs are also present in the nucleus, localizing widely in both the heterochromatin and euchromatin regions (*11*), implying the presence and operation of an IP_3-dependent Ca^{2+} control mechanism in the nucleus.

In addition to the Ca^{2+}-storage function, chromogranin B has also been shown to couple to the IP_3Rs not only at the intragranular pH 5.5 but also at a near physiological pH 7.5 (19), thereby activating the IP_3R/Ca^{2+} channels at both pH

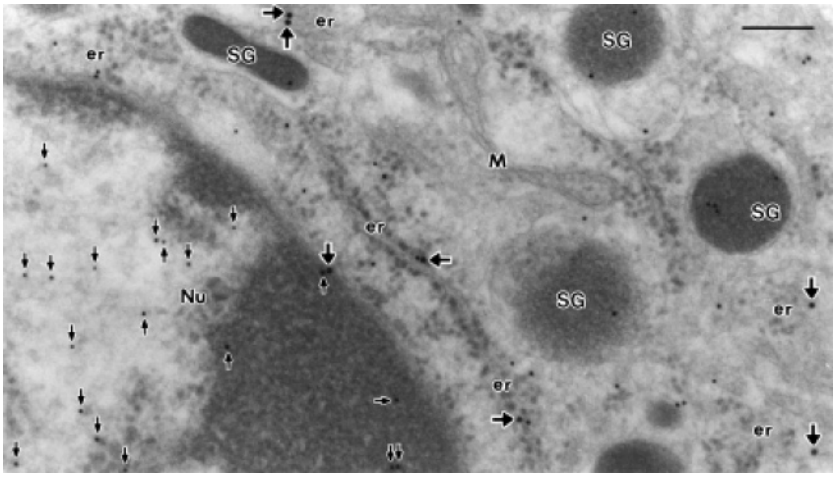

FIGURE 7: Immunogold electron microscopy showing the localization of calreticulin and CGB in adrenal chromaffin cells. Bovine adrenal medullary chromaffin cells were double immunolabeled for CGB (10 nm gold) and the ER marker calreticulin (15 nm gold). The calreticulin-labeling gold particles (marked with large arrows) are localized in the endoplasmic reticulum (er) and NE but not in the nucleoplasm. The CGB-labeling gold particles (marked with small arrows in the nucleus) are localized in secretory granules (SG), the endoplasmic reticulum (er), and nucleus (Nu). Bar = 200 nm.

(*20*). Accordingly, it has recently been demonstrated that coupling of CGB to the IP_3R/Ca^{2+} channels is essential in controlling the IP_3-dependent Ca^{2+} release from the ER (*21*), thus directly modulating the intracellular Ca^{2+} concentrations. The functional interaction of CGB with the IP_3R/Ca^{2+} channels in the ER strongly suggested the importance of this coupling in the control of IP_3-dependent intracellular Ca^{2+} concentrations of the cells in which these molecules are expressed. In light of the presence of the majority of cellular calcium in secretory granules, the ER, and nucleus, coupling of CGB to the IP_3R is likely to be a basic functional unit in IP_3-mediated Ca^{2+} release in the cell.

Analogous to the role of CGB in the ER, it is highly likely that nuclear CGB couples to the IP_3Rs in the nucleoplasm and controls the IP_3-dependent Ca^{2+} release in the nucleus. The sucrose gradient centrifugation results (Figure 2) show that both CGB and the IP_3R fractionate in ~1.55 M sucrose region while histone H1 fractionates in ~1.70 M sucrose region, suggesting that the CGB−IP_3R complex has a relatively low density compared to the nucleoplasmic mass associated with histone H1. The potential presence of a CGB−IP_3R complex in the nucleus is further strengthened by gel filtration chromatography (Figure 3) and co-immunoprecipitation studies (Figure 4). The presence of a CGB−IP_3R complex in a relatively lighter density appears to accord with a small vesicular structure with an estimated size of $\sim 2-3 \times 10^7$ Da (Figure 3). The present results in Figure 5, which showed the presence of phospholipids in the CGB or IP_3R immunoprecipitate, demonstrate the presence of phospholipids in the nucleoplasmic CGB−IP_3R complex and point out the presence of an IP_3R−CGB−phospholipid complex in the nucleoplasm.

Quantitative determination of the phospholipids in the complex indicated that the phospholipid weight in the CGB or IP_3R immmunoprecipitates is approximately 65−70% (w/w) of the protein weight (Figure 5A). In view of the fact that the phospholipid weight in the original nucleoplasmic content was ~20% that of the protein and that in the CGB−IP_3R-containing fractions of the Sephacryl S-1000 column was ~30−35% of the protein weight, the phospholipid weight of ~65−70% that of the protein in the CGB or IP_3R immmunoprecipitate appeared to point to the presence of a vesicular phospholipid structure that consisted of intravesicular CGB and membranous IP_3Rs. The estimated size of $\sim 2-3 \times 10^7$ Da suggests a structure that is larger than a ribosome and composed of several molecules of CGB and IP_3R/Ca^{2+} channels.

The potential presence of a vesicular CGB−IP_3R structure in the nucleoplasm is also supported by the double-labeling immunogold results (Figure 6). In line with the coupling of the IP_3R/Ca^{2+} channels by chromogranins in secretory granules and the ER, the double-labeling immunogold results in Figure 6 show that the IP_3R-labeling gold particles (15 nm) and the CGB-labeling gold particles (10 nm) are localized next to each other in many regions of secretory granules and the ER. Likewise, the IP_3R-labeling gold particles (15 nm) and the CGB-labeling gold particles (10 nm) are also localized next to each other in the majority of regions of the nucleus, showing little difference in the CGB−IP_3R double-labeling patterns between the ER, secretory granules, and the nucleus. In many cases the two types of gold particles are localized so close to each other that there appeared to be no room for another gold particle, probably reflecting the tight physical coupling between CGB and the IP_3Rs in the ER, secretory granules, and the nucleus. Even in the regions where only CGB- or IP_3R-labeling gold particles are found, the dominant incidence of CGB−IP_3R colocalization appears to strongly suggest the presence of coupled CGB−IP_3R. Moreover, the predominant presence of CGB and IP_3R in the heterochromatin region compared to that in the euchromatin region (Tables 1−3) appears to indicate an active participation of the IP_3R−CGB−phospholipid complex in the control of Ca^{2+} concentrations in the heterochromatin region. Moreover, the absence of the ER marker calreticulin in the nucleoplasm where nuclear CGB is localized (Figure 7) clearly indicates that the nucleoplasmic complex, which consisted of the IP_3Rs, CGB, and phospholipids, is different from the reported nucleoplasmic reticulum Ca^{2+} store (*10*).

Consistent with the present results, the nucleus is known to contain a phosphoinositide signaling system of its own, including molecules such as phosphatidylinositol 4,5-bis-

phosphate, phospholipase C, IP_3, and diacylglycerol (*12*−*16*). Existence of these molecules and the phosphoinositide signaling system indicates in nucleo production of signaling molecules IP_3 and DAG, which suggests the presence and operation of signaling systems involving these molecules in the nucleus. Further, nuclear IP_3 has been shown to release Ca^{2+} in nucleo through the IP_3R/Ca^{2+} channels, thereby stimulating the fusion of nuclear vesicles in the NE assembly (*3*). Blocking of Ca^{2+} releases through the IP_3R/Ca^{2+} channels also inhibited the fusion of nuclear vesicles (*3*), underscoring the importance of the IP_3-induced Ca^{2+} releases in nuclear processes. Despite the demonstrated presence of all three isoforms of IP_3Rs in the nucleoplasm (*11*), the question of where the calcium that can be released through the IP_3R/Ca^{2+} channels is stored in the nucleoplasm still remained. In this respect, a vesicular nucleoplasmic Ca^{2+} store that is composed of the IP_3R/Ca^{2+} channel, Ca^{2+}-storage protein, and phospholipids would be able to store and release Ca^{2+} in the nucleus, thereby controlling the intranuclear Ca^{2+} concentrations. The released nuclear Ca^{2+} could also be sequestered by this putative nucleoplasmic Ca^{2+} store, without invoking the need to be pumped out or be removed through the nuclear pore complex.

Recently, 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+} per every 20−30 nucleotides (*2*). 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 (*2*). Decondensed chromosomes were shown to contain smaller amounts of Ca^{2+} whereas condensed chromosomes contained larger amounts (*2*).

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. 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. In this respect, the present results provide the molecular basis for the IP_3-induced Ca^{2+} mobilization in the nucleoplasm. Accordingly, our preliminary results indeed showed that the putative nucleoplasmic Ca^{2+} stores rapidly release Ca^{2+} in response to inositol 1,4,5-trisphosphate. Other inositol phosphate analogues such as inositol 1,4-bisphosphate, inositol 1,3,4-trisphosphate, and inositol 1,3,4,5-tetrakisphosphate, were without effect, thus underscoring the possibility that this IP_3R−CGB−phospholipid structure serves as an IP_3-sensitive nucleoplasmic Ca^{2+} store.

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