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Localization and projected role of phosphatidylinositol 4-kinases IIα and IIβ in inositol 1,4,5-trisphosphate-sensitive nucleoplasmic Ca^{2+} store vesicles

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Abbreviations: PI, phosphatidylinositide; PI4KII, type II PI 4-kinase; CGB, chromogranin B; IP_3R, inositol 1,4,5-trisphosphate receptor

Phosphatidylinositol (PI) kinases are key molecules that participate in the phosphoinositide signaling in the cytoplasm. Despite the accumulating evidence that supports the existence and operation of independent PI signaling system in the nucleus, the exact location of the PI kinases inside the nucleus is not well defined. Here we show that PI4-kinases IIα and IIβ, which play central roles in $PI(4,5)P_2$ synthesis and PI signaling, are localized in numerous small nucleoplasmic vesicles that function as inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$)-sensitive Ca^{2+} stores. This is in accord with the past results that showed the localization of PI4(P)5-kinases that are essential in $PI(4,5)P_2$ production and $PI(4,5)P_2$ in nuclear matrix. Along with $PI(4,5)P_2$ that also exists on the nucleoplasmic vesicle membranes, the localization of PI4-kinases IIα and IIβ in the nucleoplasmic vesicles strongly implicates the vesicles to the PI signaling as well as the $Ins(1,4,5)P_3$-depenent Ca^{2+} signaling in the nucleus. Accordingly, the nucleoplasmic vesicles indeed release Ca^{2+} rapidly in response to $Ins(1,4,5)P_3$. Further, the $Ins(1,4,5)P_3$-induced Ca^{2+} release studies suggest that PI4KIIα and IIβ are localized near the $Ins(1,4,5)P_3$ receptor ($Ins(1,4,5)P_3R$)/Ca^{2+} channels on the Ca^{2+} store vesicle membranes. In view of the widespread presence of the $Ins(1,4,5)P_3$-dependent Ca^{2+} store vesicles and the need to fine-control the nuclear Ca^{2+} concentrations at multiple sites along the chromatin fibers in the nucleus, the existence of the key PI enzymes in the $Ins(1,4,5)P_3$-dependent nucleoplasmic Ca^{2+} store vesicles appears to be in perfect harmony with the physiological roles of the PI kinases in the nucleus.

Introduction

Of the many phosphatidylinositol (PI) kinases that participate in the PI cycles of the cell, PI4-kinase IIs (PI4KIIs) that exist in α- and β-forms[1-3] and PI(4)P5KIs that exist in three isoforms (α-, β-, and γ) are among the most widely studied.[4-7] PI4KIIs are the key enzymes in producing $PI(4,5)P_2$ ($PtdIns(4,5)P_2$) that is known to play a number of important roles in the cytoplasm in addition to its being an indispensable phospholipid component of cell membranes. As such, the function of PI kinases is generally discussed in the context of the cytoplasm. Nonetheless, PI kinases are also reported to be present inside the nucleus, along with phospholipase C (PLC), $PtdIns(4,5)P_2$ and $Ins(1,4,5)P_3$.[8-17] The presence of PI kinases and inositol phosphates inside the nucleus implies the existence and function of PI signaling system in the nucleus.[18,19] Further, in view of the fact that phosphatidylinositol compounds are normally components of membranous structures it becomes of immediate interest to identify the subnuclear organelles in which these molecules are part of. Yet the exact location of the molecules that participate in the phosphoinositide signaling of the nucleus still largely remains to be unclear although nonmembranous nuclear matrix complex termed "nuclear speckles" are sometimes referred to.[20-22] In particular, the intranuclear location of $PtdIns(4,5)P_2$, which is a key member of phospholipid cell membranes, appears to pose a serious challenge.[23]

We have demonstrated previously the existence of numerous small $Ins(1,4,5)P_3$-sensitive nucleoplasmic Ca^{2+} store vesicles that contain the $Ins(1,4,5)P_3Rs$ and $PtdIns(4,5)P_2$ in the nucleus.[24,25] The small $Ins(1,4,5)P_3$-sensitive nucleoplasmic Ca^{2+} store vesicles have an average diameter of ~50 nm and rapidly release Ca^{2+} in response to $Ins(1,4,5)P_3$.[25] No other inositol phosphates including inositol 1,4-bisphosphate (IP_2), inositol 1,3,4-trisphoshate, and inositol 1,3,4,5-tetrakisphosphate (IP_4) can release Ca^{2+} from these vesicles, whereby demonstrating the inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$)-specific nature of the Ca^{2+} stores. Yet the $Ins(1,4,5)P_3$ specificity would not have

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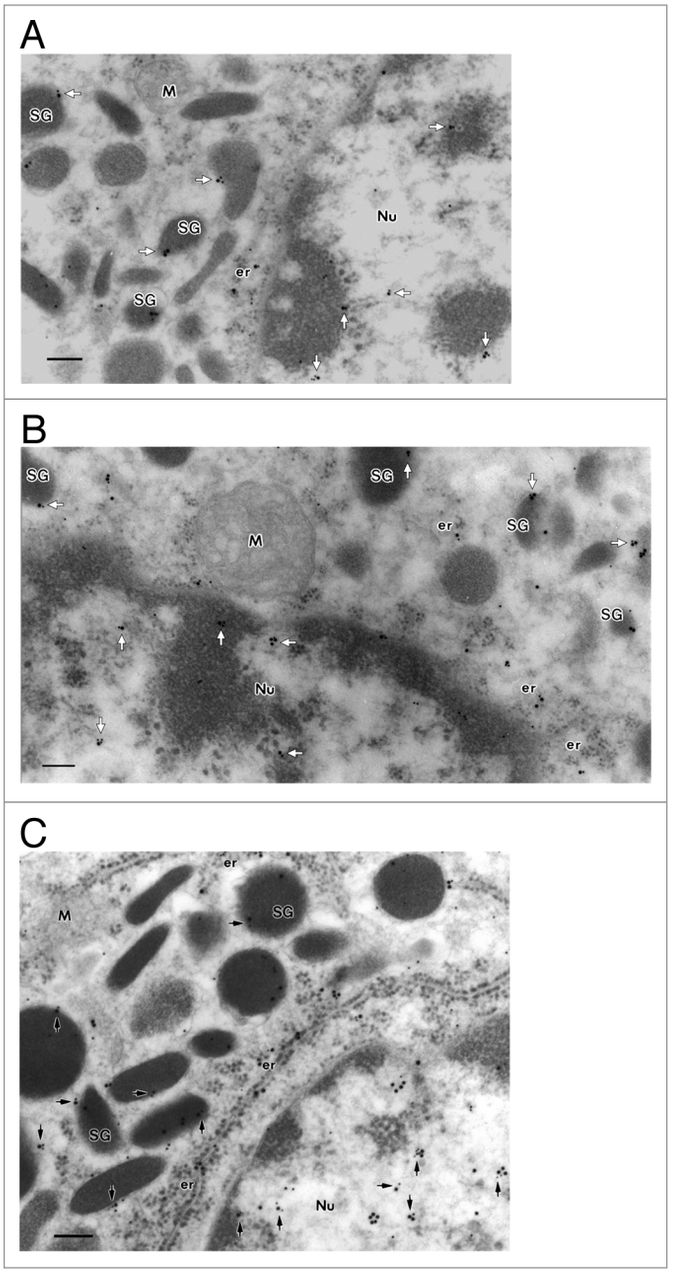

Figure 1. Immunocytochemical localization of PI4KIIα and IIβ, Ins(1,4,5)P_3Rs, and chromogranin B in adrenal chromaffin cells. Bovine adrenal chromaffin cells were immunolabeled for (**A**) PI4KIIα (10 nm gold) and Ins(1,4,5)P_3R2 (15 nm gold) with PI4KIIα and Ins(1,4,5)P_3R2 antibodies, respectively, (**B**) PI4KIIβ (10 nm gold) and Ins(1,4,5)P_3R2 (15 nm gold) with PI4KIIβ and Ins(1,4,5)P_3R2 antibodies, respectively, and (**C**) chromogranin B (15 nm gold) and Ins(1,4,5)P_3R1 (10 nm gold) with chromogranin B (CGB) and Ins(1,4,5)P_3R1 antibodies, respectively. The gold particles are localized in secretory granules (SG), endoplasmic reticulum (er), and nucleus (Nu), but not in mitochondria (M). Colocalization of respective molecules is indicated by arrows. Experiments with other Ins(1,4,5)P_3R isoforms in each set gave identical results. Bar = 200 nm.

been possible without the presence of integral membrane protein Ins(1,4,5)P_3 receptor (Ins(1,4,5)P_3R)/Ca^{2+} channels in the vesicle membranes through which Ca^{2+} is released.

Although it is not known where the PI kinases exist in the nucleus, it appears rather apparent that their location in the nucleoplasm will be limited to some form of membrane-containing structures given that most of PI4KIIα and ~30% of PI4KIIβ exist as integral membrane proteins in the cytoplasm due to palmitoylation of their cysteine residues.[26-28] In light of the fact that the Ins(1,4,5)P_3-sensitive nucleoplasmic Ca^{2+} store vesicles are the only identified intranuclear organelle with membrane bilayers,[25] we have here investigated the possibility of PI4KIIs existing in the nucleoplasmic Ca^{2+} store vesicles and found indeed the localization of PI4KIIs in these vesicles. The Ins(1,4,5)P_3-induced Ca^{2+} release studies further suggested close association of PI4KIIs with the Ins(1,4,5)P_3R/Ca^{2+} channels on the membranes of the nucleoplasmic Ca^{2+} store vesicles.

Moreover, the product of PI kinase activity PtdIns(4,5)P_2[8,9,12-15] has also been shown to exist in the PI4KII-containing nucleoplasmic Ca^{2+} store vesicles. Considering that the role of PI4KIIs is to help synthesize the membrane phospholipid PtdIns(4,5)P_2, which in turn is supposed to supply Ins(1,4,5)P_3 for Ins(1,4,5)P_3-dependent Ca^{2+} signaling mechanisms inside the nucleus, the existence of PI4KIIs in the Ins(1,4,5)P_3-sensitive nucleoplasmic Ca^{2+} store vesicles appears to be a natural consequence, shedding new light on the roles of PI kinases in the nucleus.

Results

Localization of PI4KIIs in small nucleoplasmic vesicles

To determine the exact location of PI4KIIs in the nucleus, the localization of PI4KIIα in bovine adrenal chromaffin cells was studied first with immunogold electron microscopy using the PI4KIIα-specific antibody (**Fig. 1A**). In addition, in view of the presence of integral membrane protein Ins(1,4,5)P_3Rs in the Ins(1,4,5)P_3-sensitive nucleoplasmic Ca^{2+} store vesicle membranes[25] the localization of Ins(1,4,5)P_3R isoforms was also examined using each Ins(1,4,5)P_3R isoform-specific antibody (**Fig. 1A**). **Figure 1A** shows that PI4KIIα and Ins(1,4,5)P_3R are localized not only in secretory granules but also in what appear to be the same organelles in the nucleoplasm. Given the exclusive localization of the Ins(1,4,5)P_3Rs in the Ins(1,4,5)P_3-sensitive nucleoplasmic Ca^{2+} store vesicles,[25] this result in turn raises the possibility of the localization of PI4KIIα in the Ins(1,4,5)P_3-sensitive nucleoplasmic Ca^{2+} store vesicles.

Similarly, the localization of PI4KIIβ and Ins(1,4,5)P_3Rs in bovine adrenal chromaffin cells was also examined with immunogold double-labeling electron microscopy (**Fig. 1B**). **Figure 1B** shows that PI4KIIβ and Ins(1,4,5)P_3R are localized not only in secretory granules but also in what appear to be the same organelles in the nucleoplasm, thereby further strengthening the possibility of the presence of PI4KIIβ in the Ins(1,4,5)P_3R-containing Ins(1,4,5)P_3-sensitive nucleoplasmic Ca^{2+} store vesicles. Since the Ca^{2+} storage protein chromogranin B (CGB)[29] is known to colocalize with the Ins(1,4,5)P_3Rs exclusively in the Ins(1,4,5)P_3-sensitive nucleoplasmic Ca^{2+} store vesicles,[25] the localization of CGB and the Ins(1,4,5)P_3Rs in the

nucleus was also investigated here using CGB and Ins(1,4,5)P_3R double-labeling electron microscopy (**Fig. 1C**). In line with the previous reports, Ins(1,4,5)P_3R and CGB appeared to localize in the same organelles in the nucleoplasm as they colocalized in secretory granules (**Fig. 1C**), which strengthens the possibility of the localization of the PI4KIIα and IIβ in the CGB-containing Ins(1,4,5)P_3-sensitive nucleoplasmic Ca^{2+} store vesicles.

To confirm the localization of Ins(1,4,5)P_3Rs and CGB in the Ins(1,4,5)P_3-sensitive nucleoplasmic Ca^{2+} store vesicles, the nucleoplasmic Ca^{2+} store vesicles were purified from the nuclei of bovine adrenal chromaffin cells and the expression of both CGB and Ins(1,4,5)P_3Rs was investigated using double immunogold electron microscopy (**Fig. 2A**). **Figure 2A** shows that CGB and all three Ins(1,4,5)P_3R isoforms are localized in the small nucleoplasmic vesicles as had been demonstrated before,[25,29] which in turn suggests the localization of PI4KIIα and IIβ in the membranes of the Ins(1,4,5)P_3-sensitive nucleoplasmic Ca^{2+} store vesicles. Hence, the possibility of PI4KIIα and IIβ localization in the CGB-containing Ins(1,4,5)P_3-sensitive nucleoplasmic Ca^{2+} store vesicles was further investigated using the purified nucleoplasmic Ca^{2+} store vesicles (**Fig. 2B**). Agreeing with the presence of PI4KIIα in the Ins(1,4,5)P_3-sensitive nucleoplasmic Ca^{2+} store vesicles, PI4KIIα and CGB were shown to colocalize in the purified nucleoplasmic vesicles (**Fig. 2B**). Likewise, PI4KIIβ and CGB were also shown to colocalize in the purified nucleoplasmic vesicles (**Fig. 2B**), thereby confirming the localization of PI4KIIα and IIβ in the CGB-, and Ins(1,4,5)P_3R-containing Ins(1,4,5)P_3-sensitive nucleoplasmic Ca^{2+} store vesicles.

Identification of the small nucleoplasmic vesicles as the Ins(1,4,5)P_3-sensitive Ca^{2+} store

In keeping with the expression of the Ins(1,4,5)P_3R/Ca^{2+} channels and the Ca^{2+} storage protein CGB in the vesicles, the small nucleoplasmic vesicles are known to rapidly release Ca^{2+} in response specifically to inositol 1,4,5-trisphosphate, and no other inositol phosphates exert any effect.[25] To further corroborate whether the PI4KIIα- and IIβ-containing nucleoplasmic vesicles function as the Ins(1,4,5)P_3-sensitive Ca^{2+} stores, we have tested the Ins(1,4,5)P_3-dependent Ca^{2+}-release properties (**Fig. 3**). As shown in **Figure 3**, the PI4KIIα- and IIβ-containing nucleoplasmic vesicles rapidly released Ca^{2+} in response to inositol 1,4,5-trisphosphate (**Fig. 3A**). Yet Ins(1,4,5)P_3 failed to induce Ca^{2+} release from the vesicles in the presence of the Ins(1,4,5)P_3R antibody (**Fig. 3B**) or heparin (**Fig. 3C**) while preimmune serum and IgG were without any effect on the Ins(1,4,5)P_3-induced Ca^{2+} release (**Fig. 3D**). Moreover, fitting with the nucleoplasmic nature of the Ca^{2+} store vesicles, thapsigargin, antimycin A, and oligomycin that are known to inhibit Ca^{2+} uptake into the endoplasmic reticulum and mitochondria, did not affect the Ins(1,4,5)P_3-mediated Ca^{2+} release properties from the nucleoplasmic Ca^{2+} store vesicles.

Localization of PtdIns(4,5)P_2 on the Ins(1,4,5)P_3-sensitive nucleoplasmic vesicle membranes

The existence of PI4KIIα and IIβ in the Ins(1,4,5)P_3-sensitive nucleoplasmic Ca^{2+} store vesicles strongly suggests the possibility of PtdIns(4,5)P_2 production on the vesicle membranes by

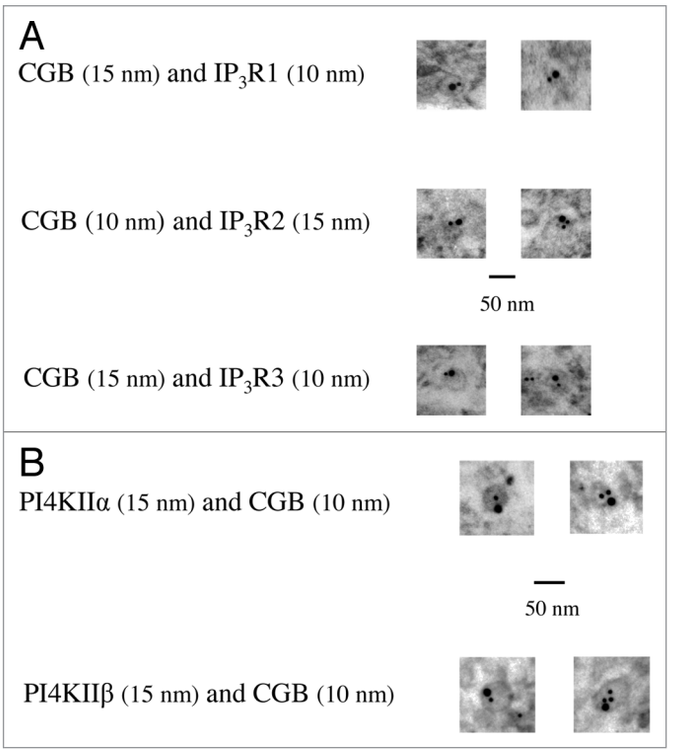

Figure 2. Immunogold labeling of the purified nucleoplasmic vesicles with chromogranin B, Ins(1,4,5)P_3Rs, PI4KIIα and IIβ. (**A**) Localization of CGB and the Ins(1,4,5)P_3Rs in the purified nucleoplasmic vesicles was examined by double-labeling immunogold electron microscopy using antibodies for CGB and each Ins(1,4,5)P_3R isoform. The size of gold particles used is shown in each set of results. The CGB-labeling gold particles are localized primarily inside the vesicles whereas the Ins(1,4,5)P_3R-labeling gold particles are in the membrane region of the vesicles. (**B**) Localization of PI4KIIα and IIβ (both 15 nm gold) and CGB (10 nm gold) was also examined by double-labeling immunogold electron microscopy. The PI4KII-labeling gold particles are primarily in the membrane region of the vesicles whereas the CGB-labeling gold particles are inside the vesicles. Bar = 50 nm.

PI4KIIα and IIβ. Since PtdIns(4,5)P_2 is the source of Ins(1,4,5)P_3 and PLC is also available in the nucleus,[9,11,18,30] it would be a natural course of event for Ins(1,4,5)P_3 to be produced from the PtdIns(4,5)P_2 of the vesicle membranes and open the nucleoplasmic Ca^{2+} store vesicle Ins(1,4,5)P_3R/Ca^{2+} channels to induce Ca^{2+} release. Underscoring this possibility and confirming the past results,[24,25] PtdIns(4,5)P_2 was shown to exist in the nucleoplasm (**Fig. 4A**). Moreover, the specific location of the PtdIns(4,5)P_2 existence in the nucleus was confirmed to be the CGB-containing Ins(1,4,5)P_3-sensitive nucleoplasmic Ca^{2+} store vesicles (**Fig. 4B**).[25] Hence, based on the information currently available a model of the Ins(1,4,5)P_3-sensitive nucleoplasmic Ca^{2+} store vesicles could be drawn as shown in **Figure 5**. In light of the fact that the nucleoplasmic vesicles have an average diameter of ~50 nm,[25] the surface area of the vesicle membranes would not be large so that the distance between molecules on the membranes is likely to be relatively short, thus allowing interactions between many of these molecules. In particular, given the large size of the Ins(1,4,5)P_3R/Ca^{2+} channels with the estimated molecular

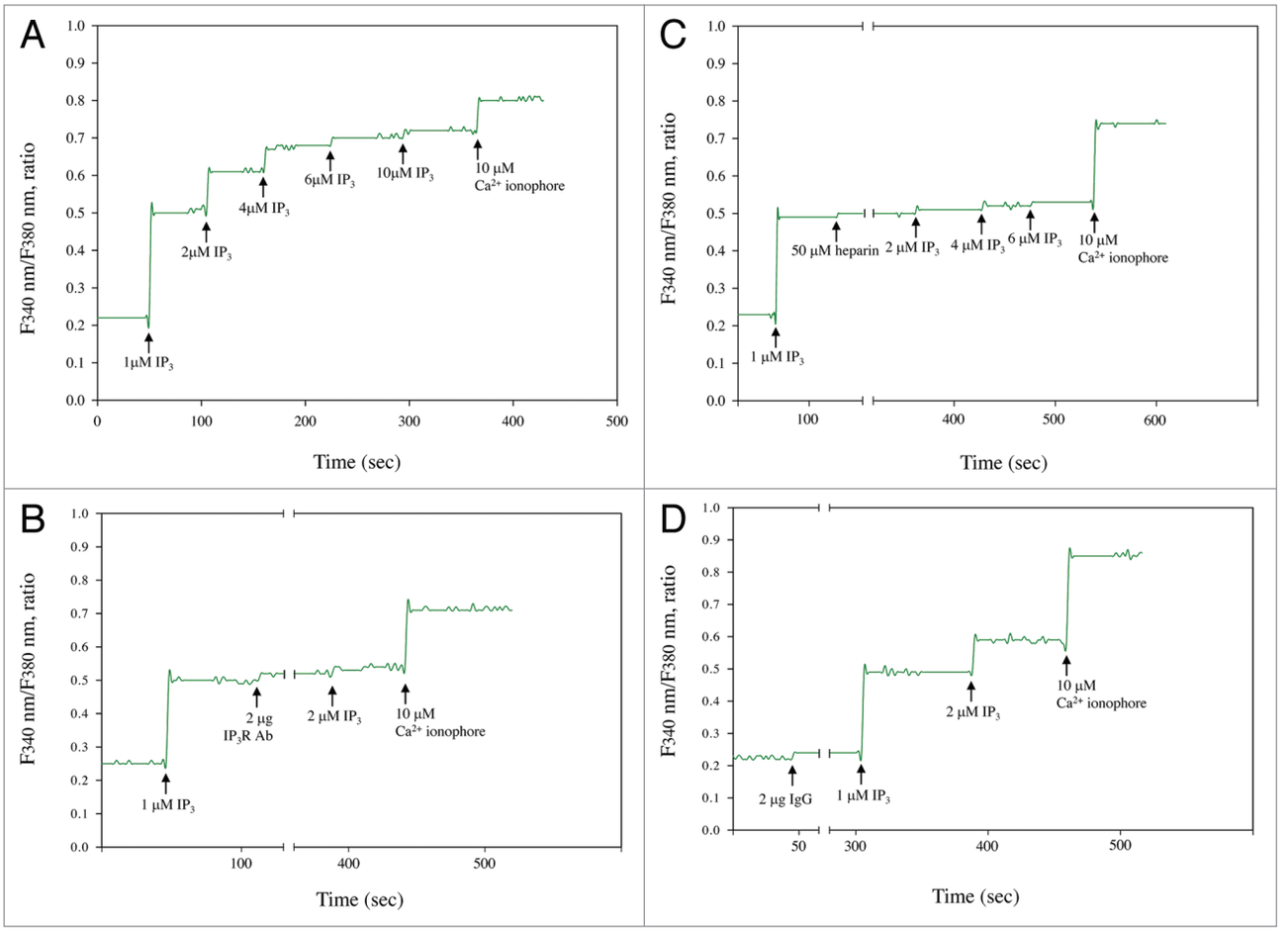

Figure 3. Ins(1,4,5)P_3-induced Ca^{2+} release from the purified nucleoplasmic vesicles. (**A**) The Ca^{2+} releases were recorded after a series of incremental additions of 1–4 μM inositol 1,4,5-trisphosphate (Ins(1,4,5)P_3) to the isolated nucleoplasmic vesicles (200 μl, 1.5 μg protein/μl) containing 20 μM fura-2. Each indicated concentration of Ins(1,4,5)P_3 represents the amount of cumulative Ins(1,4,5)P_3 added, and a cumulative total of 10 μM Ins(1,4,5)P_3 in 5 μl was added. (**B**) Addition of 1 μM inositol 1,4,5-trisphosphate (Ins(1,4,5)P_3) to the nucleoplasmic vesicles was followed by 2 μl (1 μg/μl) of affinity purified Ins(1,4,5)P_3R antibody. (**C**) Addition of 1 μM inositol 1,4,5-trisphosphate (Ins(1,4,5)P_3) was followed by the Ins(1,4,5)P_3R/Ca^{2+} channel antagonist heparin. (**D**) Addition of 2 μl (1 μg/μl) of IgG was followed by the addition of inositol 1,4,5-trisphosphate. The data shown are representatives of similar results repeated 7–10 times.

mass of ~1.2 × 10^6 daltons,[31] it is likely that the Ins(1,4,5)P_3R/Ca^{2+} channels interact with many proteins of the nucleoplasmic vesicle membranes. Furthermore, the intravesicular Ca^{2+} storage protein CGB is not only known to couple to the Ins(1,4,5)P_3R/Ca^{2+} channels and activate the Ca^{2+} channels[32,33] but also thought to interact with another Ca^{2+} storage protein SgII.[34]

Localization of PI4KIIs next to the Ins(1,4,5)P_3Rs in the Ins(1,4,5)P_3-sensitive nucleoplasmic vesicles

In order to determine whether the newly identified PI4KIIα and IIβ stay in close association with the Ins(1,4,5)P_3R/Ca^{2+} channels on the nucleoplasmic Ca^{2+} store vesicle membranes, we have performed the Ins(1,4,5)P_3-induced Ca^{2+} release experiments by microinjection of Ins(1,4,5)P_3 into the nucleus of both chromaffin and PC12 cells in the presence of the antibodies specific for PI4KIIα and PI4KIIβ (**Fig. 6**). The results in **Figure 6A** show that the antibodies for PI4KIIα and PI4KIIβ reduced the Ins(1,4,5)P_3-induced Ca^{2+} releases inside the nucleus of bovine chromaffin cells by ~38–40% while the antibodies for Ins(1,4,5)P_3R and PtdIns(4,5)P_2 reduced the Ins(1,4,5)P_3-mediated Ca^{2+} releases by ~59% and ~35%, respectively (**Fig. 6B**). However, IgG alone was without any effect on the Ins(1,4,5)P_3-induced releases in the nucleus. Similarly, the antibodies for PI4KIIα and PI4KIIβ reduced the Ins(1,4,5)P_3-induced Ca^{2+} releases inside the nucleus of PC12 cells by ~38–39% whereas IgG did not affect the Ins(1,4,5)P_3-mediated Ca^{2+} release (**Fig. 6C and D**). These results suggest that not only PtdIns(4,5)P_2 but PI4KIIα and IIβ as well exist in close association with the Ins(1,4,5)P_3R/Ca^{2+} channels on the nucleoplasmic Ca^{2+} store vesicle membranes.

Discussion

The present results not only show that PI4KIIα and IIβ exist in the nucleus but also identify the organelle in which they are localized, i.e., the Ins(1,4,5)P_3-sensitive nucleoplasmic Ca^{2+} store

vesicles. Although past studies pointed out the existence of PI kinases and the PI-based signaling systems in the nucleus,[8-19] the exact identity of the nucleoplasmic organelles in which the PI kinases exist remained unclear.[20-23] Considering that most PI4KIIα and a substantial portion of PI4KIIβ exist as membrane proteins in the cytoplasm owing to the palmitoylated cysteine residues,[26-28] it appears inevitable that the nuclear PI4KIIα and IIβ also exist as membrane proteins in the nucleus. Moreover, in view of the fact that the small numerous Ins(1,4,5)P_3-sensitive nucleoplasmic Ca^{2+} store vesicles are the only known nucleoplasmic entity with membranes,[25,29] the localization of PI4KIIα and IIβ in the nucleoplasmic vesicles is in accord with their biochemical properties.

In addition, the nucleoplasmic Ca^{2+} store vesicles not only contain the high capacity, low affinity Ca^{2+} storage proteins chromogranin B and secretogranin II but also are loaded with the Ins(1,4,5)P_3R/Ca^{2+} channels.[25,29] The high capacity, low affinity Ca^{2+} storage proteins chromogranin B and secretogranin II bind 30–50 mol of Ca^{2+}/mol of protein with dissociation constants of 2.2–3.1 mM at the near physiological pH 7.5.[29] Moreover, CGB is coupled to the Ins(1,4,5)P_3Rs and activates the Ins(1,4,5)P_3R/Ca^{2+} channels, increasing the mean open time and the open probability of the Ca^{2+} channels, 24-fold and 8-fold, respectively.[32] Yet despite the seeming similarity between the cytoplasmic secretory granules and the nucleoplasmic Ca^{2+} store vesicles in having both the Ins(1,4,5)P_3R/Ca^{2+} channels on their respective membranes and the coupled CGB inside the vesicles, the Ins(1,4,5)P_3R/Ca^{2+} channels in the nucleoplasmic vesicles are at least 3- to 4-fold more sensitive to Ins(1,4,5)P_3 than those of secretory granules.[35] This marked difference in the sensitivity of the Ins(1,4,5)P_3R/Ca^{2+} channels to Ins(1,4,5)P_3 appears to be due to the difference in the molecular structures of secretory granules and the nucleoplasmic vesicles.

Although secretory granules function as the major Ins(1,4,5)P_3-sensitive Ca^{2+} store in the cytoplasm of secretory cells,[36,37] the organelle's primary physiological role in secretory cells is to store high concentrations of a variety of secretory cargoes and transport them to the site of secretion. Accordingly, secretory granules of bovine chromaffin cells contain >500 mM catecholamines, ~150 mM ATP, 40 mM Ca^{2+}, and 2–4 mM chromogranins, in addition to many other molecules.[38-40] Secretory granules from other types of cells also contain high concentrations of molecules specific to each type of granules that they look electron-dense under an electron microscope. In contrast, the nucleoplasmic Ca^{2+} store vesicles appear transparent under an electron microscope, clearly highlighting the difference in the nucleoplasmic vesicular contents from those stored in secretory granules albeit the Ca^{2+} concentration in the nucleoplasmic vesicles is expected to be high.

Given the storage of high concentrations of hormones and other molecules besides the granin proteins in secretory granules,[40-42] the Ins(1,4,5)P_3R/Ca^{2+} channels on secretory granule membranes are likely to interact with multiple molecules that may complicate the channel-activating roles of the coupled CGB. In contrast, the nucleoplasmic Ca^{2+} store vesicles appear to be specialized organelles that exist primarily for the control of nucleoplasmic Ca^{2+} concentrations, free from duties such as storage and transport of heavy loads of secretory cargoes. In this respect, the nucleoplasmic vesicles are likely to contain only the molecules that are necessary for them to function as the Ins(1,4,5)P_3-sensitive nucleoplasmic Ca^{2+} stores, i.e., the Ins(1,4,5)P_3R/Ca^{2+} channels, Ca^{2+} storage and activating proteins CGB and SgII, and probably high concentrations of Ca^{2+} (**Fig. 5**), which are the minimum requirement for the nucleoplasmic vesicles to function as the Ins(1,4,5)P_3-sensitive Ca^{2+} stores in the nucleus. The transparent appearance of the vesicles under an electron microscope appears to suggest a rather simple molecular organization suitable for the role as specialized Ins(1,4,5)P_3-sensitive Ca^{2+} stores. Hence, the ~4-fold higher sensitivity of the nucleoplasmic Ins(1,4,5)P_3R/Ca^{2+} channels to Ins(1,4,5)P_3 compared with those of secretory granules[35] is deemed to result from the unhindered activating effects of coupled CGB on the Ins(1,4,5)P_3R/Ca^{2+} channels of the nucleoplasmic vesicles.

Yet given the dynamic and multiple Ca^{2+} needs inside the nucleus, the mere existence of only the Ins(1,4,5)P_3R/Ca^{2+} channels, Ca^{2+} storage proteins, and Ca^{2+} in the nucleoplasmic vesicles would not be sufficient for the organelle to adequately

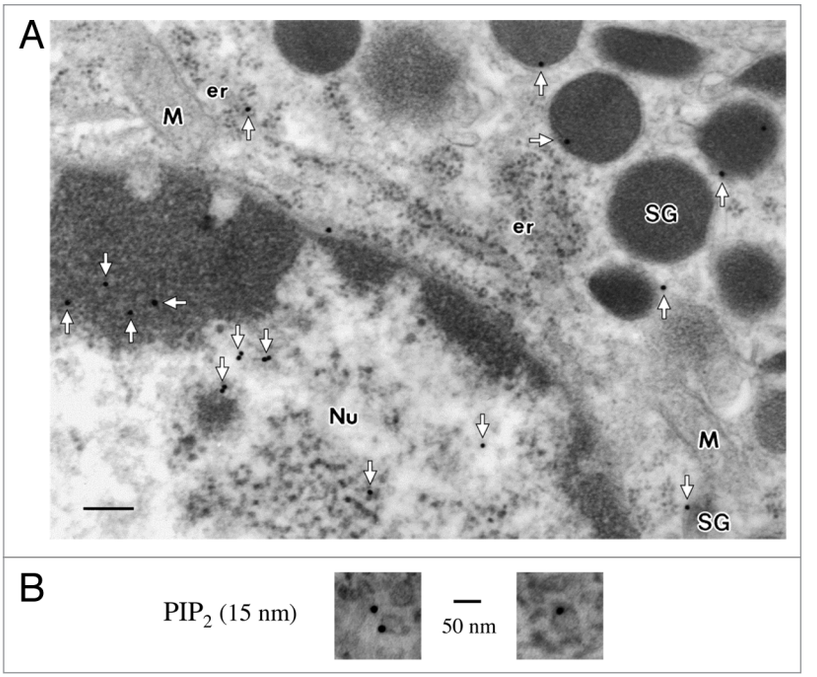

Figure 4. Immunogold labeling of adrenal chromaffin cells and the purified nucleoplasmic vesicles with PtdIns(4,5)P_2. Bovine adrenal chromaffin cells (**A**) and purified Ins(1,4,5)P_3-sensitive nucleoplasmic vesicles (**B**) were immunolabeled for PtdIns(4,5)P_2 (15 nm gold). (**A**) The PtdIns(4,5)P_2-labeling gold particles are localized in secretory granules (SG), endoplasmic reticulum (er), and nucleus (Nu), but not in mitochondria (M). The PtdIns(4,5)P_2-labeling gold particles are indicated by arrows. Bar = 200 nm. (**B**) PtdIns(4,5)P_2 is shown to localize on the membranes of the Ins(1,4,5)P_3-sensitive nucleoplasmic vesicles. Bar = 50 nm.

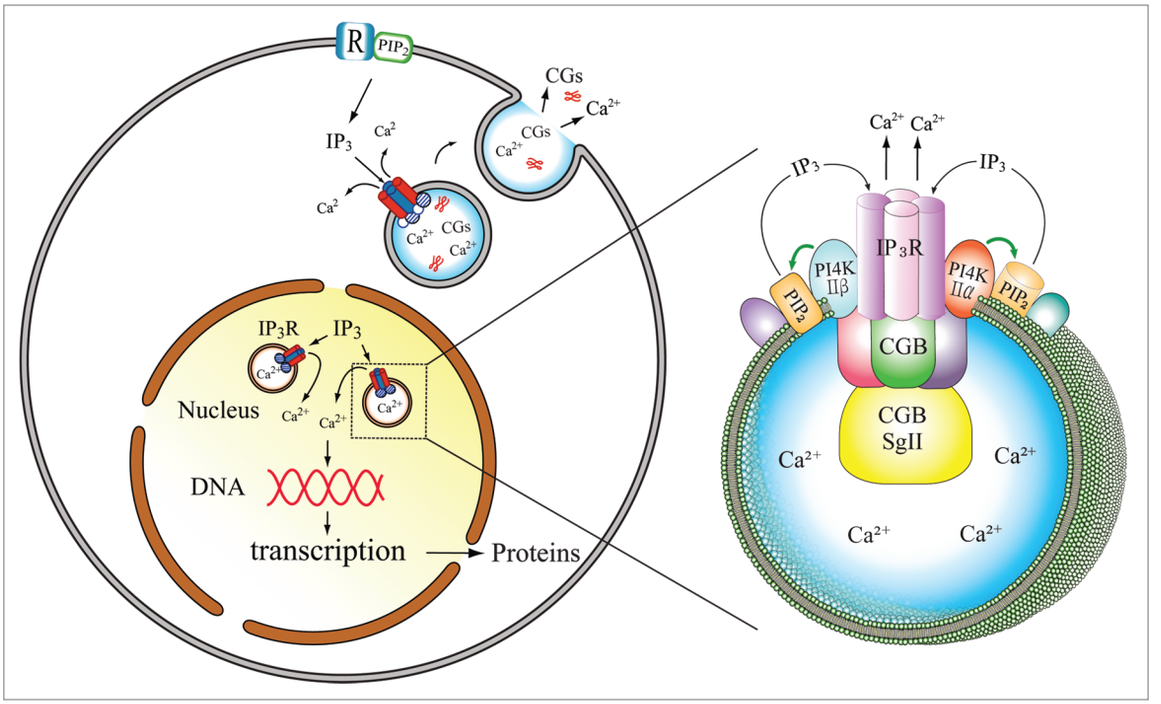

Figure 5. Schematic drawing of the Ins(1,4,5)P_3-sensitive nucleoplasmic Ca^{2+} store vesicle. The Ins(1,4,5)P_3-sensitive nucleoplasmic Ca^{2+} store vesicle is drawn with the nucleus and the whole cell showing secretory granules and the molecules identified in the nucleoplasmic vesicle, i.e., the Ins(1,4,5)P_3R/Ca^{2+} channel, chromogranin B (CGB), secretogranin II (SgII), PI4KIIα and IIβ, PtdIns(4,5)P_2, and large amounts of Ca^{2+}. Chromogranin B is not only known to couple to the Ins(1,4,5)P_3R/Ca^{2+} channels and activate the channels[32,33] but also thought to interact with SgII. In addition, many other unidentified molecules, potentially including phospholipase C and other PI kinases, are thought to be associated with the vesicle.

coordinate the supply of Ins(1,4,5)P_3 for the control of Ins(1,4,5)P_3-dependent nuclear Ca^{2+} concentrations. Furthermore, considering that Ins(1,4,5)P_3 is produced from PtdIns(4,5)P_2, and PI4KIIα and IIβ play central roles in producing PtdIns(4,5)P_2, the nuclear Ca^{2+} control role of the nucleoplasmic Ca^{2+} store vesicles would not be possible without a close mechanistic correlation with the PI cycling systems inside the nucleus. It seems therefore very natural that PI4KIIα and IIβ are localized in the Ins(1,4,5)P_3-sensitive nucleoplasmic Ca^{2+} store vesicles. Moreover, the reported presence of PI(4)P5Ks, one of the two key enzymes in PtdIns(4,5)P_2 production, in the nucleus seems to underscore the pivotal importance of PtdIns(4,5)P_2 in nuclear functions. Interestingly, the PI(4)P5KIα and its product PtdIns(4,5)P_2 are suggested to take part in transcription control of certain types of genes through their interaction with poly(A) polymerase termed 'Star-PAP' in the nucleus.[21,43] Recent studies also indicate the presence of PI(5)P and its metabolizing enzyme PI(5)P4Ks in the nucleus,[12,14,16,44,45] thereby demonstrating the widespread expression of a variety of PI kinases in the nucleus. In this respect, the widespread existence of PI kinases that participate in PtdIns(4,5)P_2 production in the nucleus appears to further raise the possibility of localization of even PI(4)P5Ks and PI(5)P4Ks in the Ins(1,4,5)P_3-sensitive nucleoplasmic Ca^{2+} store vesicles. Furthermore, the results in **Figure 6** indicate that PI4KIIα and PI4KIIβ, along with PtdIns(4,5)P_2, are localized near the Ins(1,4,5)P_3R/Ca^{2+} channels (**Fig. 5**). The apparent close localization of PI4KIIα and IIβ near the Ins(1,4,5)P_3R/Ca^{2+} channels and PtdIns(4,5)P_2 on the membranes of the nucleoplasmic vesicles (**Fig. 5**) appears to ensure the Ins(1,4,5)P_3-dependent nuclear Ca^{2+} control role of the nucleoplasmic Ca^{2+} store vesicles in tune with the PI cycle of the nucleus. In this regard, the localization of PI(4)P5Ks and PtdIns(4,5)P_2 inside the nucleus as evidenced in fluorescence microscopy[16,17,22,23,46] is in accord with the present results.

Further, given that PtdIns(4,5)P_2 on the membranes of the Ins(1,4,5)P_3-sensitive nucleoplasmic Ca^{2+} store vesicles can be replenished by the PI4KIIα and PI4KIIβ, the localization of the PI kinases near the Ins(1,4,5)P_3R/Ca^{2+} channels accords well with the projected function of the vesicles in the control of Ca^{2+} storage and concentrations inside the nucleus. Moreover, in our unpublished results the Ins(1,4,5)P_3-sensitive nucleoplasmic Ca^{2+} store vesicles were shown to stay in close contact with many nucleosomes in the nucleoplasm (Yoo SH, unpublished results), which implied the Ca^{2+} control role of the nucleoplasmic Ca^{2+} store vesicles in chromatins. Considering that there are numerous small Ins(1,4,5)P_3-sensitive nucleoplasmic Ca^{2+} store vesicles in the nucleus,[24,25] the presence of the Ca^{2+} store vesicles in close association with the chromatins suggests the vesicles' intimate involvement in the Ca^{2+} control roles at multiple sites along the chromatin fibers, which is also in line with the previously suggested roles of nuclear phosphoinositides.[9,19] In this regard, the presence of PI4KIIs in the small nucleoplasmic Ca^{2+} store vesicles will be of critical importance not only in the Ins(1,4,5)P_3-dependent Ca^{2+} control function of the nucleoplasmic Ca^{2+}

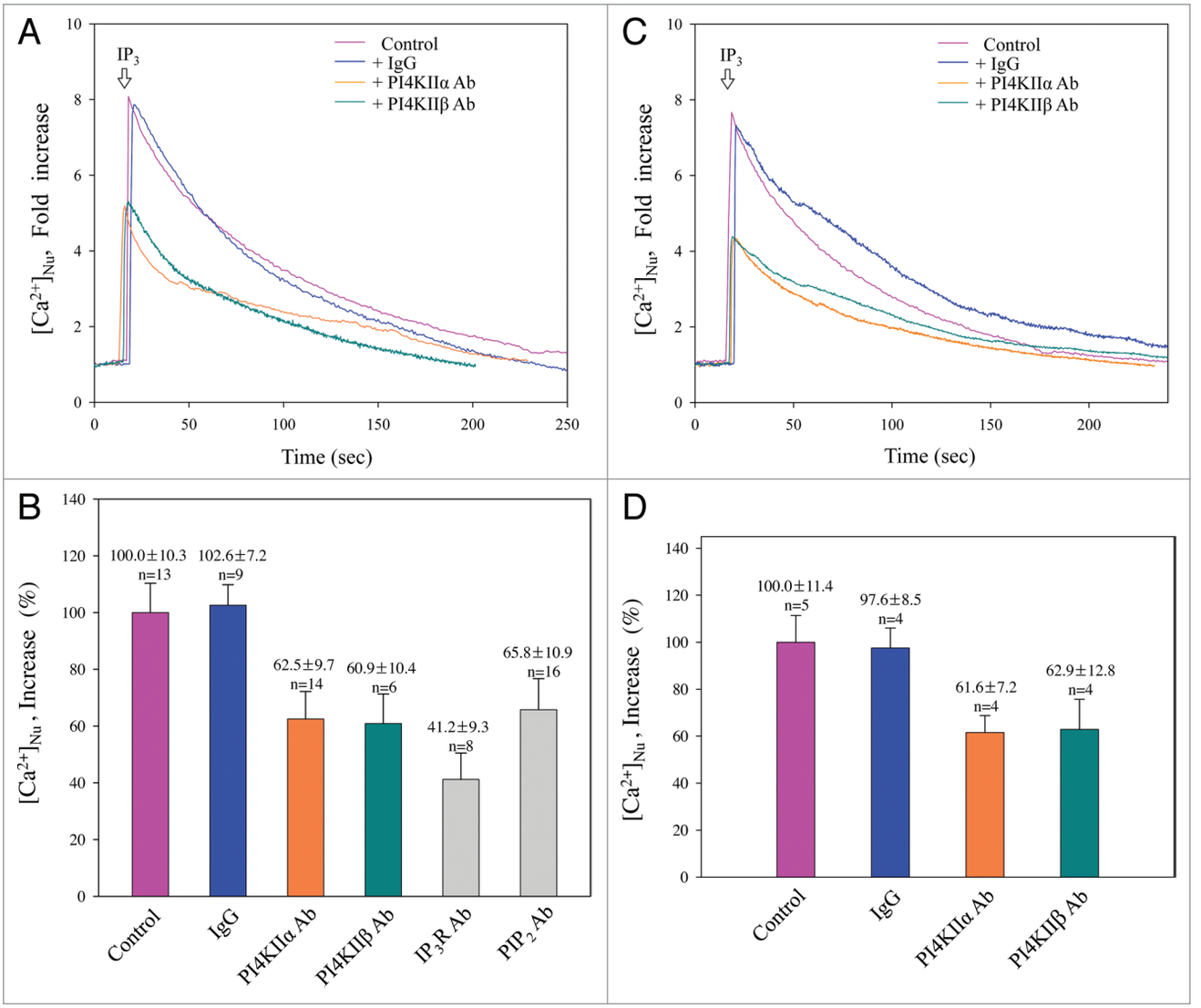

Figure 6. Microinjection of Ins(1,4,5)P_3 into the nucleus of bovine chromaffin and PC12 cells and Ca^{2+} imaging. Ten nM Ins(1,4,5)P_3 was microinjected into the nucleus of bovine chromaffin (**A**) and PC12 cells (**C**) at the time indicated by a downward arrow, and the resulting Ca^{2+} release images are shown as a function of time in pseudo colors. The effect of the various antibodies on the Ca^{2+} release was determined after microinjection of each antibody (2 μg) into the nucleus first, followed by microinjection of 10 nM Ins(1,4,5)P_3 as described in the Experimental Procedures section. The results shown are typical of bovine chromaffin (**A**) and PC12 cells (**C**), and the Ca^{2+} release results for chromaffin (**B**) and PC12 cells (**D**) are also expressed in bar graphs (mean ± s.d., n equals the number of cells tested).

store vesicles but also in the maintenance and operation of the PI cycles in the nucleus.

Being the home of chromosomes and chromosomes containing billions of negative charges in the form of one negative charge per nucleotide, the nucleus must contain large amounts of positive charges just to neutralize the high magnitude of intrinsic negative charges and maintain an appropriate nuclear structure. This extraordinary need for positive charges in chromosomes is primarily met by high concentrations of Ca^{2+}, ranging from ~20 mM when the chromosomes are in relaxed state to ~32 mM when in condensed state,[47] though Mg^{2+} and histones are also known to supply positive charges. Although the Ca^{2+} concentrations stored in the Ins(1,4,5)P_3-sensitive nucleoplasmic Ca^{2+} store vesicles are not known at present, it is highly likely that they contain large amounts of Ca^{2+}, probably in high mM range, given the presence of ~11 mM Ca^{2+} in the nuclei of bovine chromaffin cells[25] and the presence of high capacity, low affinity Ca^{2+} storage proteins CGB, and SgII in the nucleoplasmic vesicles.[29]

That the chromosomes maintain millimolar range of Ca^{2+} concentrations inside the nucleus simply translates into the existence and operation of high capacity nucleoplasmic Ca^{2+} stores that are capable of controlling the millimolar Ca^{2+}. Moreover, in light of the need to fine-control the Ca^{2+} needs at multiple sites along the chromatin fibers that are building blocks of chromosomes, it is inevitable that the nuclear Ca^{2+} stores should be small enough to fit in between the chromatins and be widely present along the chromatin fibers. In this respect, the

Ins(1,4,5)P_3-sensitive nucleoplasmic Ca^{2+} store vesicles appear to be the organelle that satisfies not only the physical requirements to be small and widespread (**Figs. 1 and 2**)[25] but also the physiological needs to store and release Ca^{2+} in tune with the PI cycles inside the nucleus. Therefore, the existence of the key enzyme in the PI cycles PI4KIIs in the nucleoplasmic Ca^{2+} store vesicles is in perfect harmony with the Ca^{2+} storage and control role of the Ins(1,4,5)P_3-sensitive nucleoplasmic Ca^{2+} store vesicles and appears to highlight the under-studied and yet essential roles of PI kinases in the nucleus.

Materials and Methods

Antibodies

Polyclonal anti-rabbit CGB antibody was raised against intact recombinant CGB, and the specificity of the antibody was confirmed.[48-50] The monoclonal anti-mouse CGB antibody (L1BF2) that recognizes an epitope within residues 526–575[25] was raised against intact bovine CGB. The polyclonal anti-rabbit PI4KIIα and IIβ antibodies (generous gift of Dr Joseph Albanesi of The University of Texas Southwestern Medical Center at Dallas, Texas) were raised against peptides corresponding to the N-terminal residues 2–17 of rat PI4KIIα and 2–15 of human PI4KIIβ, respectively. The polyclonal antibodies were affinity purified on each immobilized peptide, and their specificities have been confirmed.[28,51,52] Ins(1,4,5)P_3R peptides specific to terminal 10–13 amino acids of type 1 (HPPHMNVNPQQPA), type 2 (SNTPHENHHMPPA), and type 3 (FVDVQNCMSR) were synthesized with a C-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.[48,53] In addition, an Ins(1,4,5)P_3R peptide (DEEEVWLFWR DSNKEI) with an approximate consensus sequence with all three Ins(1,4,5)P_3R isoforms was synthesized with a C-terminal cysteine, and the anti-rabbit polyclonal antibody was prepared[54] as described for the isoform-specific Ins(1,4,5)P_3R antibodies. Monoclonal antibodies for PI(4,5)P_2 were from Echelon Biosciences (catalog number, Z-A045) and Assay Designs (Enzo Life Sciences; ADI-915-052), and both antibodies gave identical results.

Preparation of 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 3 layers of cheesecloth and centrifuged for 15 min at 1000 x 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 x g. The pellet that contained the nuclei was resuspended in 60 ml of buffer 2, homogenized with a Teflon pestle, and 3 ml of the homogenates were layered over 28 ml of sucrose gradient (1.4–2.2 M) for centrifugation at 98 000 x 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 x 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 x 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 brief sonication, followed by centrifugation at 21 000 x g for 30 min. Highly pure nucleoplasm was obtained in the supernatant of this run. All the procedures were performed at 4 °C.

Purification of the Ins(1,4,5)P_3-sensitive nucleoplasmic Ca^{2+} store vesicles

The extracted nucleoplasm (sample #1) from bovine chromaffin cells was first fractionated by Sephacryl S-1000 gel filtration chromatography as described.[24] The fractions containing CGB and the Ins(1,4,5)P_3Rs were pooled and concentrated (sample #2). The concentrated sample #2 was further fractionated by sucrose gradient centrifugation. For this 7 mg of the nucleoplasmic proteins in 3 ml 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 x 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 that contained the Ins(1,4,5)P_3Rs and CGB were pooled and used as the purified nucleoplasmic Ca^{2+} store vesicles. To prepare the nucleoplasmic vesicles for electron microscopy and Ca^{2+} release study, the purified nucleoplasmic vesicles were 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 nucleoplasmic vesicles to aggregate so that they could be pelleted by centrifugation at 21 000 x g for 3 min for subsequent use in electron microscopy and Ca^{2+} release measurements.

Immunogold electron microscopy

For the immunogold electron microscopy of chromaffin cells and the purified nucleoplasmic vesicles, the tissue samples from bovine adrenal medulla as well as the pelleted nucleoplasmic vesicle samples were prepared on Formvar/carbon-coated nickel grids as described.[49] 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 and PtdIns(4,5)P_2 antibodies or polyclonal CGB, PI4KIIα, PI4KIIβ, and Ins(1,4,5)P_3R antibodies 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 (or 15)-nm gold-conjugated goat anti-mouse IgG or IgM, diluted in solution A. For double immunogold labeling experiment, the grids that had gone through the first-labeling step with the first antibody were reacted once more with the second antibody and labeled with 15 (or 10)-nm gold particles. Controls for the specificity of each antibody-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 antibody-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 JEOL 1011 electron microscope.

Measurements of Ca^{2+} release from the Ins(1,4,5)P_3-sensitive nucleoplasmic Ca^{2+} store vesicles by fluorescence microscopy

To prepare the purified nucleoplasmic vesicles for Ca^{2+} release experiments, the freeze-thawed nucleoplasmic vesicles (2.5–3.0 mg protein/1 ml buffer 3) were pelleted by centrifugation at 21 000 x g for 3 min. The nucleoplasmic vesicles were 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 the nucleoplasmic vesicle 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 vesicle solution, fura-2 was added at a final concentration of 20 μM and 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. The Ca^{2+} release from the nucleoplasmic vesicles was analyzed by dual excitation of fura-2 at 340nm and 380nm with a LAMBDA LS xenon arc lamp and LAMBDA 10–2 optical filter changer (Sutter Instrument Co). The emission fluorescence signals at 510 nm were collected using a band pass filter of D510/40 nm (Chroma Technology Corp) and Hamamatsu C4742-95 digital CCD camera. The ratio images were acquired every second and continued with successive addition of inositol 1,4,5-trisphosphate and other test molecules. The changes of fluorescence ratio at the two-excitation wavelengths were calculated by MetaFluor software (Universal Imaging Corporation). 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 sulphoxide.

Isolation and primary culture of bovine adrenal chromaffin cells

Bovine adrenal glands supplied by the local slaughterhouse were processed within 1 h after death of the animals. The adrenal glands were washed with Locke's buffer (154 mM NaCl, 5.6 mM KCl, 3.6 mM $NaHCO_3$, 5.6 mM glucose, 5 mM HEPES, pH 7.4) through the adrenal vein and then infiltrated for 15 min at 37 °C with Locke's buffer containing 0.2% collagenase and 0.5% BSA, followed by additional infiltration with buffer change. The adrenal medullae were dissected free of the cortex, finely minced, and incubated in collagenase-containing Locke's buffer for 30 min at 37 °C in spinner flask. The cells were dissociated by filtering through 250 μm sterile nylon mesh and centrifuged at 2800 x g for 10 min. The pellet was then resuspended in Locke's buffer and filtered through 100 μm nylon cell strainer (BD Falcon). After this, the chromaffin cell-containing solution was placed on Percoll (Sigma-Aldrich Co) and centrifuged at 20 000 x g for 20 min. The chromaffin cell-containing middle layer was filtered through 40 μm nylon cell strainer, and the filtrate was suspended with 300 ml of Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, followed by centrifugation at 1000 x *g* for 10 min. The chromaffin cell pellet was then resuspended in DMEM and centrifuged again as above, after which highly purified and Percoll-free chromaffin cells were obtained in the pellet. Finally, the isolated chromaffin cells were suspended in DMEM supplemented with 10% FBS, and 2×10^5 cells were plated on a glass coverslip (22 × 22 mm) coated with collagen type IV (BD Biosciences), and cultured for 1–2 d at 37 °C, 5% CO_2 atmosphere before Ca^{2+} release studies.

Cell culture and preparation for Ca^{2+} release studies

PC12 cells were maintained in RPMI 1640 (Gibco BRL) medium supplemented with 10% fetal bovine serum, and ~1×10^5 PC12 cells were plated on a collagen-coated glass coverslip in a well containing 800 μl of RPMI 1640 medium supplemented with 10% FBS before Ca^{2+} release studies. For real-time Ca^{2+} release studies, bovine chromaffin cells and PC12 cells grown on coverslips were stabilized with serum free medium (OPTI-MEM I) for 30 min, and then were loaded with Fluo-4, AM (Molecular Probes) at a final concentration of 4 μM in OPTI-MEM I for 40 min at 37 °C, 5% CO_2. After incubation, the cells were washed 3 times with OPTI-MEM I, followed by stabilization with the same medium for 30 min at room temperature. Then, each coverslip containing the cells was mounted on a perfusion chamber on the stage of an inverted microscope (IX71, Olympus), and the cells that had been uniformly loaded with Fluo-4 in the nucleus and cytosol were selected for microinjection and for [Ca^{2+}] measurements.

Microinjection of Ins(1,4,5)P_3 and antibodies

Microinjections of Ins(1,4,5)P_3 to the bovine chromaffin and PC12 cells were done with an Eppendorf system (Injectman NI2 5181, Femtojet 5247; Eppendorf-Netheler-Hinz) using pipettes (~100 nm inner diameter) pulled from quartz glass (outer diameter, 1.0 mm; inner diameter, 0.7 mm, Sutter Instrument) using a P-2000 micropipette puller (Sutter Instrument). The Ins(1,4,5)P_3 and antibodies to be microinjected were diluted to their final concentrations in buffer (20 mM Hepes, 110 mM KCl, 2 mM $MgCl_2$, 5 mM KH_2PO_4, and 10 mM NaCl, pH 7.2) and filtered (0.2 μm) directly before filling into microinjection pipette (~100 nm in inner diameter). Injections were made using the semiautomatic mode of the Eppendorf system at a pipette angle of a 45° and under the following instrument settings: injection pressure 80 hPa, compensatory pressure 60 hPa, injection time 0.5 s, and velocity of the pipette 2000 μm/sec.

Under such conditions using Femtotips II (~500 nm inner diameter) as pipettes, the injection volume had previously been estimated to be 1–1.5% of the cell volume in the case of Jurkat T-lymphocytes.[55] Hence, in light of the similarity in size between Jurkat T-lymphocytes and PC12 cells, and much larger bovine chromaffin cells that have an average diameter ~1.5 to 2 times larger than that of PC12 cells, our injection volume was also expected to be ~1% of the PC12 cell volume or less than 0.3% of the bovine chromaffin cell volume. The Ins(1,4,5)P_3-induced Ca^{2+} release was initiated by the microinjection of 10 nM Ins(1,4,5)P_3 directly into the nucleus and the changes in the fluorescence Ca^{2+} images were acquired every 100 ms. In the

course of successive microinjection of antibodies and Ins(1,4,5)P_3, the movement associated with the first injection of antibodies (including anti-PI4KIIα, -PI4KIIβ, -Ins(1,4,5)P_3R, -PtdIns(4,5)P_2, and IgG) into the nucleus of the cells caused transient increases in [Ca^{2+}]. Hence, subsequent microinjection of Ins(1,4,5)P_3 to the same region was performed 5 min later, allowing the [Ca^{2+}] to return to the basal level and the microinjected antibodies to react with their antigens.

Detection of nuclear Ca^{2+} signals with confocal microscopy

The confocal images of intranuclear Ca^{2+} signals of bovine chromaffin and PC12 cells were recorded near the middle of the nucleus using a Perkin Elmer Ultra*View* LCI confocal imaging system with 60×, 1.4 NA objective lens. To detect the confocal fluorescence images of the calcium signals, fluo-4 was excited at 488 nm using an argon laser and a 488/10 nm excitation filter (Chroma Technology Corp), and the emission fluorescence signals were collected through a HQ525/50 nm band pass filter. Images were acquired every 100 ms after microinjection of 10 nM Ins(1,4,5)P_3, and the Ca^{2+} release in the nucleus of microinjected cells was measured using the Ultra*VIEW* LCI confocal imaging system with 100× objective (NA = 1.35) from the optical Z-section transverse the middle region of the nucleus of the cell. The baseline fluorescence (F_o) of each ROI was calculated as the average fluo-4 fluorescence intensity of 100 frames before Ins(1,4,5)P_3 injection. The onset of the Ca^{2+} signal was determined as the time point at which F- F_o began to rise above 5% of the difference between F_{max} - F_o for the first time.

Disclosure of Potential Conflicts of Interest

No potential conflict of interest was disclosed.

Acknowledgments

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