Functional Implication of Neuronal Calcium Sensor-1 and Phosphoinositol 4-Kinase-β Interaction in Regulated Exocytosis of PC12 Cells*

Received for publication, September 7, 2005, and in revised form, April 24, 2006. Published, JBC Papers in Press, April 25, 2006, DOI 10.1074/jbc.M509842200

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Several studies have shown that the neuronal calcium sensor (NCS-1) and phosphoinositol 4-kinase-β (PI4K-β) regulate the exocytic process of nerve and neuroendocrine cells. The aim of our study was to investigate their possible interaction at rest and during stimulation in living cells and to decipher the role of this interaction in the secretory process. In PC12 cells, we observed a stimulation-induced recruitment of NCS-1 and PI4K-β from the intracellular compartment toward the plasma membrane. This recruitment was highly correlated to the intracellular Ca2⁺ rise induced by secretagogues. Using fluorescence resonance energy transfer between PI4K-β-EYFP and NCS-1-EYFP, we show that both proteins are interacting in resting cells and that this interaction increases with stimulation. It appears that the membrane insertion of NCS-1 is necessary for the interaction with PI4K-β, since a mutation that prevented the membrane insertion of NCS-1 abolished NCS-1-PI4K-β interaction, as revealed by fluorescence resonance energy transfer analysis. Additionally, the overexpression of mutated NCS-1 prevents the stimulatory effect on secretion induced by PI4K-β, suggesting that the interaction of the two proteins on a membrane compartment is necessary for the secretory function. Moreover, mutation of endogenous PI4K-β by small interfering RNA inhibits secretion and completely prevents the stimulatory effect of NCS-1 on calcium-evoked exocytosis from permeabilized PC12 cells, showing directly for the first time the functional implication of a NCS-1-PI4K-β complex in regulated exocytosis.

Exocytosis in nerve cells and in neuroendocrine cells is mediated by the exocytotic fusion of synaptic vesicles and secretory granules (dense core vesicles) with the plasma membrane in a process that is largely governed by Ca2⁺. Formation of the SNARE complex between the vesicular synaptobrevin and the plasma membrane syntaxin and SNAP-25 is generally accepted to play a major role in Ca2⁺-triggered exocytosis. Although the neuronal calcium sensor (NCS-1) (1) orthologue of frequenin in invertebrates, and phosphoinositol 4-kinase-β (PI4K-β) (2) have been shown to play an important role in regulating synaptic vesicle and secretory granule exocytosis, the underlying molecular mechanisms are still not fully understood. These proteins are mostly found in the cytosol but are also found on the membrane of secretory vesicles (1, 3). NCS-1 has been shown to modulate Ca2⁺ membrane conductance through a direct effect on Ca2⁺ channels (2, 4, 5). Indeed, the C-terminal part of NCS-1 interacts directly with the β subunit of P/Q type voltage-gated calcium channels (5), and a C-terminal peptide of NCS-1 inhibited the facilitation current through P/Q calcium channels after NCS-1 injection (4). On the other hand, PI4K-β together with phosphoinositide 5-kinase produces phosphoinositol 4,5-diphosphate (PIP₂), which can form phospholipid microdomains (6–8). Besides its effect on calcium channels, NCS-1 has also been shown to regulate PI4K-β activity (2, 9). Indeed, PI4K-β and NCS-1 have been shown to interact in vitro (10), in Madin-Darby canine kidney (11), COS-7 (12), and PC12 cells (13). It was first believed that NCS-1 undergoes a Ca2⁺-induced conformational change, which allows its membrane insertion in secretory granules through its myristoyl residue and the subsequent interaction with PI4K-β (14, 15). However, this model has been recently challenged (16, 17), since the myristoyl group of NCS-1 was reported to be freely accessible, and the membrane association of NCS-1 was independent of intracellular Ca2⁺ concentration.

The aim of our study was to determine the conditions of NCS-1 and PI4K-β interaction in living cells and to correlate this interaction with the secretory function. For this purpose, we used living transfected PC12 cells overexpressing PI4K-β-EYFP and/or NCS-1-EYFP as fusion proteins. A stimulation-induced recruitment of NCS-1 and PI4K-β was observed by biochemical and immunofluorescence approaches as well as by time lapse fluorescence imaging and correlated with the intracellular Ca2⁺ concentration changes. The interaction between NCS-1 and PI4K-β in PC12 cells was demonstrated through fluorescence resonance energy transfer (FRET) between two chromophores and by immunoprecipitation. Finally, extinction experiments demonstrated the functional implication of the NCS-1-PI4K-β interaction in dense core secretory granule exocytosis.

EXPERIMENTAL PROCEDURES

Materials—Poly-L-ornithine (M, 30,000–70,000) and the anti-BCP were from Sigma, and monoclonal anti-NCS-1 antibody was purchased from BD Biosciences. Rabbit anti-human PI4K-β type III antibody was obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). Anti-SNAP-25 antibody was from Chemicon International (Temecula, CA).
Culture medium (RPMI 1640), laminin, nerve growth factor (2.5 S), and horse serum were purchased from Invitrogen. Fetal calf serum was purchased from Dominique Dutscher S.A. (Brumath, France), and type I collagen from rat tail was a product from BD Biosciences. Fura-2-AM was from Molecular Probes, Inc. (Eugene, OR). The transfection reagent (GenePorter) was from Ozyme (St. Quentin/Yvelines, France).

Cell Culture and Transfection Procedure—PC12 cells were plated on 35-mm glass bottom plastic Petri dishes (Falcon) coated with collagen and polyornithine (0.1 mg/ml each). They were grown in RPMI 1640 medium containing L-glutamine supplemented with 10% horse serum, 5% fetal calf serum (both decomplemented), 50 units/ml penicillin, and 50 μg/ml streptomycin. They were cultivated for 7 days in a humidified 5% CO2 atmosphere at 37 °C. To induce cell differentiation, 2.5 S murine nerve growth factor (50 ng/ml) was added to the culture medium 24 h after plating.

For transfection experiments, we used the following constructs. NCS-1-EYFP construct was generated by subcloning the rat NCS-1 cDNA into pEYFP-N1 (Clontech), as previously described (18), and the PI4K-β-ECFP construct was generated by subcloning the human PI4K-β cDNA into pECFP-C2 (Clontech), retaining its kinase activity. After 4 days in culture, 0.5 μg of plasmid of interest was transfected using 5 μl of GenePorter in 1 ml of RPMI 1640 without serum and antibiotics. For co-transfection experiments, 0.5 μg of each plasmid was added to the cells with 10 μl of GenePorter in 1 ml of RPMI 1640. After 4–5 h of incubation, the transfection medium was replaced by full culture medium supplemented with murine nerve growth factor. The cells were used 24–36 h after transfection.

Co-transfection efficacy was assessed by spectral fluorescence recording (see below) of single cells. In every co-transfected cell displaying fluorescence, two peaks at 495 and 525 nM were observed. The transfection efficacy for NCS-1-EYFP expression was also compared in monotransfected cells and in co-transfected cells. The fluorescence intensity from single cells (excitation, 460 nm; emission, 525 nm) was 4 times less in co-transfected cells (1090 ± 200, n = 20) than in mono-transfected cells (4280 ± 770, n = 20), indicating that co-transfection was effective but affected the level of NCS-1-EYFP expression.

For small interfering RNA (siRNA) experiments, a bicistronic mammalian expression vector directing the synthesis of GH and siRNAs was effective but affected the level of NCS-1-EYFP expression. Transfected cells (4280 ± 200) were electroporated (260 V, 1060 μF, for 17 ms) in 107 PC12 cells, and 72 h post-transfection, cells were used for Western blot and immunofluorescence experiments. The transfection efficiency under these conditions was followed by 2 h of incubation with primary antibodies in PBS containing 0.1% Triton X-100™, cells were incubated for 1 h in 10% normal goat serum in PBS to block nonspecific binding. This step was followed by 2 h of incubation with primary antibodies in PBS containing 5% normal goat serum at room temperature (anti-PI4K-β, 1:100; anti-NCS-1, 1:100). Goat anti-rabbit or anti-mouse secondary antibody (1:1000 in 5% goat serum) Alexafluor 488™-conjugated (Molecular Probes) or indocarbocyanine-conjugated (Cy3; Jackson Immunoresearch, West Grove, PA) was applied for 1 h in PBS. Cultures were finally mounted in Vectashield™ medium (Vector Laboratories, Burlington, CA) to reduce photobleaching. Controls were obtained by omitting the primary antibody in the incubation bath, and no staining of the cells was observed in these conditions. Immunostaining was performed as described previously (23), and stained cells were visualized using a Zeiss LSM 510 confocal microscope. Quantification was performed using the Zeiss CSLM instrument 3.2 software. Regions of interest (ROI) were selected by taking a similar ellipse (6.5 μm2) with the great diagonal (5 μm) at the plasma membrane.

Imaging Experiments in Living Cells—PC12 cells were incubated for 30 min at 37 °C in Krebs medium (106 mM NaCl, 4.5 mM KCl, 1.2 mM MgSO4, 2.5 mM CaCl2, 11 mM d-glucose, 1.2 mM KH2PO4, 25 mM NaHCO3, pH 7.4, equilibrated with 5% CO2) containing 2 μM Fura-2-AM. Cells were then washed and subsequently incubated for 20 min with Krebs medium. After three washes, the cells were placed on an inverted microscope (Axiovert 35M; Zeiss), superfused with Krebs solution (1 ml/min), and alternatively illuminated at 350 ± 10 nm (for Fura-2) or at 490 ± 10 nm (for EGFP). The fluorescent emission was observed using a dichroic mirror at 500 nm and a long pass filter at 510 nm. For each excitation wavelength (350 nm for Fura-2 and 490 nm for EGFP) and every 2.5 s, an image was recorded using an intensified CCD camera (Extended Isis; Photonic Science) and the Fluostar software (Imstar, Paris, France). Drugs were applied by superfusion in Krebs solution for 1 min.

Image Analysis and Semiquantification—Image analysis was performed according to Dupont et al. (22). Two image series, each corresponding to one excitation wavelength (350 and 490 nm), were recorded for each experiment. After background subtraction from each image series, another image series was calculated by exponential interpolation to assess for the fluorescence base line during the whole recording period, taking into account probe bleaching and possible probe leaks. This interpolation was performed on a pixel-to-pixel basis between the initial recording period and the final recording period. Finally, a third image series was then calculated, dividing experimental images by interpolated base-line images on a pixel-to-pixel basis. This third set of images was called the normalized image series. The normalized values were independent of fluorescence bleaching and probe concentration heterogeneity in the preparation. Image analysis was performed on a DEC-Alpha work station (Digital Co., Boston, MA) using the Khoros function library (Khoral Research Inc.).

On the normalized image series of the Fura-2 fluorescence, the data were then semiquantified by defining regions of interest usually corresponding to entire cells in the microscopic field. The average of the normalized values inside these regions was calculated and plotted versus the image record time. The curves displayed peaks corresponding to stimulation-induced increases in intracellular [Ca2+]i. On the normalized image series of the EGFP fluorescence, we defined regions of interest corresponding to the periphery and the center of each fluorescent cell body in the microscopic field. For each cell, the average value of the normalized fluorescence intensity of the outer region was divided by the average value of the corresponding center. This ratio was then plotted

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versus recording time and revealed the protein translocation during the experiment.

Fluorescence Resonance Energy Transfer Measurement—For FRET experiments, we used PI4K-β/H9252 fused with ECFP as a donor and NCS-1 fused with EYFP as an acceptor. PC12 cells monotransfected with plasmids encoding PI4K-β/H9252-ECFP or NCS-1-EYFP or co-transfected with both plasmids were placed on an inverted fluorescence microscope (Axiovert 35M; Zeiss; dichroic mirror 470 nM), which was connected by optical fibers with the excitation and the emission monochromators of a spectrofluorimeter (Alphascan, PTI). A single cell was selected in the microscope field. As for imaging experiments, the cells were superfused by Krebs solution (1 ml/min), and drugs were applied by superfusion for 1 min. The cells were illuminated at 430 nM (the excitation wavelength of ECFP), and emission spectra between 480 and 600 nM were recorded every 15 s during the experiment. For FRET measurements, the emission spectra were deconvoluted after baseline subtraction to distinguish donor and acceptor fluorescence intensities. To minimize the bias due to differential expression of the tagged proteins in single cells, donor and acceptor intensities were then normalized by dividing the calculated intensities by the initial measured intensity. These normalized intensities were then plotted versus time.

For bleaching resonance energy transfer, the procedure was similar. An initial emission spectrum (excitation 430 nM, the excitation wavelength of ECFP) was recorded, and the preparation was then continuously illuminated at 480 nM (excitation wavelength of EYFP) for 10 min while superfused with Krebs solution. A second emission spectrum (excitation 430 nM) was recorded after illumination. The changes in fluorescence intensities of the donor and the acceptor were calculated after spectral deconvolution.

In separate experiments, PC12 cells coexpressing PI4K-β-ECFP and NCS-1-EYFP were fixed with 4% paraformaldehyde in phosphate-buffered saline. They were observed on a confocal microscope (Leica TCS SP2 AOBS), which allows spectral recording for each pixel of the image. The preparation was excited at 405 nM using a diode laser, and the images were spectrally recorded between 440 and 580 nM. The FRET signal was assessed by calculating the intensity ratio $I_{530}/I_{490}$. FRET images were generated using the Metamorph software (Universal Imaging).

Subcellular Fractionation—PC12 cells were washed twice with Locke's solution and then incubated for 10 min with Locke's solution (Resting) or stimulated for 10 min with an elevated K⁺ solution. Medium was removed, and cells were immediately scraped in 1 ml of
0.32 M sucrose (20 mM Tris, pH 8.0). Cells were broken in a Dounce homogenizer and centrifuged at 800 × g for 15 min. The supernatant was further centrifuged at 20,000 × g for 20 min. The resulting supernatant was further centrifuged for 60 min at 100,000 × g to obtain the cytosol (supernatant) and microsomes. The 20,000 × g pellet containing the crude membrane fraction was resuspended in 0.32 M sucrose (20 mM Tris, pH 8.0), layered on a cushion sucrose density gradient (1–1.6 M sucrose, 20 mM Tris, pH 8.0), and centrifuged for 90 min at 100,000 × g to separate the plasma membrane from secretory granules (23). The upper fractions containing SNAP-25 (plasma membrane marker) and the pellet containing chromogranin A (secretory granule markers) were collected and resuspended in buffer (20 mM Tris, pH 8.0, 100 mM NaCl, 1 mM MgCl₂) before protein quantification by Bradford measurements.

**Immunoprecipitation**—Cell extracts were prepared by lysing the cells as described previously (19). Lysates were clarified by centrifugation, and 500-μg aliquots were incubated for 24 h at 4 °C with anti-PI4K-β (1:50 dilution). PI4K-β was immunoprecipitated from the supernatant of each sample using protein A-agarose (19). Precipitated proteins were resolved on 12% polyacrylamide-SDS gels and immunoblotted with anti-PI4K-β (1:1000) and anti-NCS-1 (1:1000) antibodies. Blots were processed using the Western-Light Plus chemiluminescent detection system (Tropix, Bedford, MA).

**Secretion Measurements**—PC12 cells were co-transfected with the plasmids of interest together with pXGH5 encoding human growth hormone using GenePorter. GH release experiments were performed for 48 h after transfection (23). PC12 cells were washed twice with Locke’s solution and incubated for 10 min in Ca²⁺-free Locke’s solution (basal release) or with Locke’s solution containing either 59 mM KCl and 85 mM NaCl or 30 μM ATP. The supernatant was collected, and the cells were harvested by scraping in 10 mM phosphate-buffered saline. The amount of GH secreted into the medium or present in the cells was measured using a radioimmunoassay kit (Nichols Institute). The amount of GH secretion is expressed as a percentage of total GH present in the cells before stimulation.

**RESULTS**

**Localization of PI4K-β and NCS-1**—In resting PC12 cells, endogenous PI4K-β immunoreactivity appeared as small patches inside the cytoplasm, and the density of the fluorescence was higher around the nucleus (Fig. 1), probably in the Golgi apparatus, consistent with earlier observations (1, 3). Discrete immunopositive portions of the plasma membrane were also observed, and a careful examination showed that the immunolabel was discontinuous on these portions (Fig. 1A, arrow). In depolarized cells, immunoreactivity appeared more concentrated at the level of the plasma membrane (Fig. 1A). No fluorescence staining of the cells was observed when the primary antibody was omitted, and the distribution pattern of PI4K-β-EGFP was similar to endogenous PI4K-β-immunoreactivity, albeit slightly more fuzzy (see Figs. 2 and 6).

NCS-1 immunoreactivity was partially cytosolic with a higher density of the fluorescence near the nucleus in resting PC12 cells, although we could also observe punctuated immunopositive signal (Fig. 1A) near the plasma membrane and some accumulation of the protein in the distal part of the neurites (Fig. 1A). In depolarized cells, NCS-1 immunoreactivity also appeared increased at the periphery of the cell, mostly displaying a dotted pattern (Fig. 1A). We also compared the distribution of endogenous NCS-1 and PI4K-β in subcellular fractions prepared from resting and secretagogue-stimulated PC12 cells. As illustrated in Fig. 1B, stimulation with elevated K⁺ for 10 min, which initiates calcium-dependent exocytosis in PC12 cells, increased the amount of NCS-1 and PI4K-β detected in the plasma membrane fraction mostly at the expense of the cytosolic fraction. To corroborate these observations, we performed colocalization experiments with the plasma membrane marker SNAP-25 in resting and stimulated cells and a semiquantitative analysis of the amount of colocalized immunoreactivity (Fig. 2A). In resting conditions, 16.8 ± 1.5% and 11.0 ± 1.2% of PI4K-β and NCS-1 signals colocalized with SNAP-25 labeling, respectively. After stimulation, the degree of colocalization of PI4K-β and NCS-1 with SNAP-25 increased to 33.6 ± 1.6% and 27.4 ± 2%, respectively. These results clearly indicate a recruitment of PI4K-β and NCS-1 to the plasma membrane during stimulation.

Since the distribution of NCS-1 and PI4K-β at the plasma membrane did not appear to be uniform, a more detailed analysis was performed on smaller portions of the plasma membrane. We found that in the selected area (ROI), 29.4 ± 2.6% and 20.0 ± 2.4% of PI4K-β and NCS-1 signals colocalized with SNAP-25 labeling, respectively, in resting conditions. A 10-min high potassium stimulation resulted in a 3.2- and a 2.2-fold increase in the degree of colocalization with the plasma membrane marker SNAP-25 for NCS-1 and PI4K-β, respectively (Fig. 2A).

**Kinetics of NCS-1 and PI4K-β Recruitment during Cell Stimulation**—The redistribution of PI4K-β and NCS-1 toward the plasma membrane of depolarized cells was further studied using cells overexpressing PI4K-β-EGFP or NCS-1-EGFP. Fluorescence images of the transfected cells were time lapse-recorded during a 1-min KCl application. Pseudocolor images of normalized fluorescence clearly showed a reversible translocation of either PI4K-β-EGFP (Fig. 2B) or NCS-1-EGFP (Fig. 2C) when the cells were depolarized. This phenomenon was also revealed by calculating the ratio of outer over inner average fluorescence intensity (Fig. 2, B and C). The plots revealed a short lag phase and also showed the reversal of recruitment shortly after stimulus removal. To verify the specificity of the observed redistribution, we also used PC12 cells overexpressing EGFP alone and stimulated either by KCl or ATP application. In both cases, we did not observe a translocation of the fluorescent protein, indicating that the recruitment observed for PI4K-β-EGFP and NCS-1-EGFP was most probably not due to the fluorescent tag itself (not shown). To study the correlation between protein recruitment and changes in [Ca²⁺]ᵢ, PC12 cells overexpressing either PI4K-β-EGFP or NCS-1-EGFP were loaded with Fura-2, a fluorescent probe for [Ca²⁺]. Fluorescent images for Fura-2 and for the fluorescent protein were alternatively recorded during cell stimulation. After KCl application, the normalized fluorescence of Fura-2 increased, indicating an increase in [Ca²⁺]ᵢ during stimulation (Fig. 3). In the same cells, the membrane recruitment of either PI4K-β-EGFP (Fig. 3A) or NCS-1-EGFP (Fig. 3B) occurred with similar kinetics (see Table 1). As a control, the kinetics of the [Ca²⁺]ᵢ response was also measured in adjacent nontransfected cells. In both cases, the kinetics of the [Ca²⁺]ᵢ response in control and in transfected cells were similar (Fig. 3, A and B). We measured these kinetic parameters by calculating the time constant of the response onset and of the response decrease (Table 1). The onset was best described by a monoexponential either for Ca²⁺ response or for protein translocation, and we did not find significant differences in the time constant values (Table 1). The response decrease was best described by a biexponential, except for NCS-1-EGFP recruitment kinetics, which followed a monoexponential decay. Again we did not find significant differences on the first exponential kinetics. These data suggested that the transfection process and the overexpression of the protein did not modify the initial steps of the cellular response to stimulation and that the protein redistribution was tightly correlated to changes in [Ca²⁺]ᵢ.

PC12 cells have also been described to respond in a dose-dependent way to ATP stimulation (2). We tested the correlation between the [Ca²⁺]ᵢ response and PI4K-β or NCS-1 recruitment during a 30 μM ATP application.
Interaction of NCS-1 and PI4K-β in Living PC12 Cells

FIGURE 2. Imaging of NCS-1 and PI4K-β recruitment in KCl-stimulated PC12 cells. A, resting or stimulated PC12 cells expressing either PI4K-β-EGFP or NCS-1-EGFP were fixed and processed for staining with anti-SNAP-25 antibodies. Confocal images in the green (PI4K-β or NCS-1) or red (SNAP-25) were recorded simultaneously in the same optical section by a double exposure procedure. Masks represent the region of colocalization obtained by selecting the double-labeled pixels. Bar, 5 μm. Specific ROI of the plasma membrane were...
tion for 1 min. In this case, we also observed a good correlation between the 
ion enhancement during stimulation and the redistribution of either 
PKC- or NCS-1 (Fig. 3, D and E). This indicated that a distinct stimulus 
also induced translocation of both proteins.

Interestingly, in a recent study, Taverna et al. (13) showed that the 
interaction of PKC- and NCS-1 was dependent on NCS-1 myristoy-
lation. We thus used a mutant of NCS-1, NCS-1(2A), fused with EGFP 
and overexpressed in PC12 cells. This point mutation abolishes the 
consensus myristoylation site of NCS-1 and prevents its insertion in 
lipid membranes (12). Accordingly, we found that NCS-1(2A)-EGFP 
was cytosolic (not shown). No recruitment of the mutated protein was 
observed after KCl application (Fig. 3 C).

**Interaction of NCS-1 and PKC- in Resting Cells**—Since PKC- and 
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observed after KCl application (Fig. 3C).

**Interaction of NCS-1 and PKC- in Resting Cells**—Since PKC- and 
NCS-1 have been described to interact *in vitro* and in cells (11–13), we

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**FIGURE 3.** Correlation of PKC- or NCS-1 recruitment with intracellular Ca**
**i concentration. Transfected PC12 cells overexpressing PKC-EGFP (A and D) or NCS-1-EGFP (B and E) or NCS-1(2A)-EGFP (C) were loaded with Fura-2 to detect intracellular [Ca**
**i] changes. Cells were then alternatively illuminated at 350 and 490 nm while recording images and stimulating the cells with an 80 mM KCl application (solid bar) (A–C) or with 30 μM ATP (D and E). The kinetics of protein translocation (filled squares) (calculated as described in the legend to Fig. 2) are similar to intracellular [Ca**
**i] changes (open squares). Cells not expressing the fusion proteins in the same preparations were taken as controls (open circles); the kinetics of [Ca**
**i] changes was identical in transfected cells and in control cells, indicating that the overexpression of either NCS-1-EGFP or PKC-EGFP did not modify the initial steps of the cellular response. In cells overexpressing NCS-1(2A)-EGFP (a mutant of NCS-1 with an alteration of its myristoylation site) (C), no protein translocation was observed. These data are representative of at least 10 cells in each condition and experiments performed on three different cell cultures.
Interaction of NCS-1 and PI4K-β in Living PC12 Cells

TABLE 1
Comparison of [Ca^{2+}] response kinetics with PI4K-β-EGFP or NCS-1-EGFP recruitment kinetics

| Cells                        | Response onset time constants ± S.E. | NCS-1-EGFP |
|------------------------------|--------------------------------------|------------|
|                              | PI4K-β-EGFP                          | NCS-1-EGFP |
|                              | \(\tau_1\)                          | \(\tau_2\) |
| Ca^{2+} control              | 18.3 ± 1.5                           | 13.6 ± 2.1 |
| Ca^{2+} -transfected         | 13.7 ± 1.4                           | 12.1 ± 1.5 |
| Protein recruitment          | 10.6 ± 9.4                           | 7.4 ± 2.0  |

|                              | Response decrease time constants ± S.E. | NCS-1-EGFP |
|------------------------------|----------------------------------------|------------|
|                              | \(\tau_1\)                          | \(\tau_2\) |
| Ca^{2+} control              | 27.7 ± 2.2                            | 50.2 ± 1.7 |
| Ca^{2+} -transfected         | 26.5 ± 3.5                            | 57.7 ± 1.5 |
| Protein recruitment          | 27.1 ± 4.3                            | 59.7 ± 3.3 |

decided to use FRET to assess this interaction in living cells. For this purpose, we chose the fluorophore pair ECFP/EYFP, which has been previously shown in several applications to act as a donor/acceptor pair with a Förster radius of ~50 Å (24). PC12 cells were cotransfected to overexpress PI4K-β-ECFP and NCS-1-EYFP as donor and acceptor fluorophores, respectively. The level of expression of each protein was estimated by measurements using an excitation wavelength of 480 nm. This showed that the fluorescence intensity of NCS-1-EYFP in optimal conditions was 200% that of PI4K-β-ECFP. When the resting cotransfected cells were excited at 430 nm (the excitation wavelength of ECFP), the emission spectrum displayed two fluorescence intensity peaks at 495 and 525 nm (Fig. 4C) characteristic of PI4K-β-ECFP and NCS-1-EYFP, respectively. The high fluorescence intensity of NCS-1-EYFP as compared with the PI4K-β-ECFP intensity prompted us to verify whether the labeled proteins were interacting in resting cells. We thus recorded a first fluorescence emission spectrum (excitation 430 nm) and then illuminated the cell at 480 nm (the excitation wavelength of EYFP) for 10 min to induce a selective bleaching of EYFP. A second fluorescence emission spectrum (excitation 430 nm) was recorded afterward. In monotransfected cells, this paradigm induced a global decrease of the fluorescence intensity (Fig. 4A and B). As expected, this decrease was small but significant for PI4K-β-ECFP and large for NCS-1-EYFP. In cotransfected cells, we observed a slight increase in PI4K-β-ECFP fluorescence and a decrease for NCS-1-EYFP (Fig. 4C). This effect was further quantified after spectral deconvolution (Fig. 4D) and showed a 16.4 ± 5.3% increase of fluorescence for PI4K-β-ECFP and a simultaneous 30 ± 3.8% decrease of NCS-1-EYFP fluorescence, quite similar to the decrease observed in NCS-1-EYFP-monotransfected cells. This phenomenon demonstrated FRET occurring between PI4K-β-ECFP and NCS-1-EYFP in resting PC12 cells with a FRET efficacy of 12.9 ± 4.8%. It suggested that in resting PC12 cells, part of PI4K-β-ECFP and NCS-1-EYFP were close enough to allow energy transfer between the fluorophores. It should be emphasized that due to the partial bleaching of the acceptor fluorophore in our paradigm, this interaction is probably underestimated. A rapid estimate suggests that the maximal FRET efficacy might be around 30% in resting cells.

To verify the specificity of the interaction described herein, we cotransfected PC12 cells with plasmids encoding NCS-1-EYFP and ECFP (Fig. 4G) or in cells co-expressing PI4K-β-ECFP and EYFP (Fig. 4H). In both cases, the fluorescence spectra (excitation 430 nm) displayed a single peak at 495 nm, and no increase of the peak was observed after the bleaching paradigm, indicating an absence of FRET signal.

The specificity of the FRET signal was further verified in PC12 cells co-expressing PI4K-β-ECFP and the mutated NCS-1-(2A)-EYFP. In this case, although both proteins were expressed in the co-transfected cells, the fluorescence spectrum (excitation 430 nm) exhibited a single peak at 495 nm, and no increase of this peak intensity was observed after the bleaching paradigm (Fig. 4F). This result demonstrated that the interaction between NCS-1 and PI4K-β observed in resting PC12 cells was specific and that it required myristoylation of NCS-1.

Interaction of NCS-1 and PI4K-β during Stimulation—The question arose whether the interaction between PI4K-β and NCS-1 was stable or could be modulated by cell stimulation. To answer this question, fluorescence emission spectra (excitation 430 nm) of single cells coexpressing PI4K-β-ECFP and NCS-1-EYFP were recorded every 15 s, whereas the cells were depolarized by a 1-min application of 80 mM KCl. After spectral deconvolution and normalization of the fluorescence intensity, it appeared that during stimulation, ECFP displayed a reversible decrease in fluorescence intensity, whereas that of EYFP was increased. Hence, the ratio of F(EYFP)/F(ECFP) reversibly increased (Fig. 5A). This indicated that the FRET signal between the two proteins increased during stimulation. Indeed, when the same experiment was performed with monotransfected cells overexpressing either PI4K-β-ECFP or NCS-1-EYFP, no significant changes in fluorescence intensity were observed. This was also the case in cells co-expressing either NCS-1-EYFP and ECFP or PI4K-β-ECFP and EYFP (not shown). We also verified that this effect was independent of the stimulation mode; when the cotransfected cells were stimulated by a 30 μM ATP application for 1 min, the ratio of NCS-1-EYFP fluorescence over PI4K-β-ECFP fluorescence displayed also a reversible and slow increase (Fig. 5B), reproducing the effect of K⁺ −induced depolarization. When the myristoylation site of NCS-1 was mutated, we observed no interaction with PI4K-β in stimulated cells. Indeed, the NCS-1 mutation of the myristoylation site completely abolished the increase of the FRET signal induced either by KCl or ATP application (Fig. 5, A and B).

The yeast homologue of NCS-1 associates with Pik1, the yeast homologue of PI4K-β-type III enzyme, and stimulates its activity (9). Similarly, NCS-1 and PI4K-β type III physically associate in neuronal cells (13) and when coexpressed in COS-7 cells (12). Therefore, to corroborate our FRET observations, we performed co-immunoprecipitation experiments to examine whether endogenous NCS-1 and PI4K-β form a complex in vivo. Antibodies to PI4K-β specifically immunoprecipitated a 110-kDa protein as revealed by Western blot. Probing the lower part of the nitrocellulose membrane with an anti-NCS-1 antibody, we found that endogenous NCS-1 coprecipitated with PI4K-β (Fig. 5C). In agreement with the increased FRET signal observed in stimulated cells, we detected more NCS-1 coprecipitated with PI4K-β from KCl-incubated cells (Fig. 5C). In agreement with our fluorescence observations, we found no communoprecipitation of the mutated NCS-1-(2A) with PI4K-β (Fig. 5D). Altogether, these experiments are in favor of the view...
**FIGURE 4.** FRET experiments showing interaction between PI4K-β-ECFP and NCS-1-EYFP in resting PC12 cells. Fluorescence emission spectra of PI4K-β-ECFP (excitation 430 nm) and NCS-1-EYFP (excitation 460 nm) were recorded in living PC12 cells (solid line). The cells were then illuminated at 480 nm for 10 min to bleach the acceptor fluorophore before recording again an emission fluorescence spectrum (dotted line). In cells transfected with either PI4K-β-ECFP (A) or NCS-1-EYFP (B), illumination induces a decrease in the fluorescence intensity (dotted line). In cells cotransfected with PI4K-β-ECFP and NCS-1-EYFP (C), illumination induces a fluorescence increase at 495 nm and a decrease at 525 nm, indicating fluorescence energy transfer between the two probes. This suggests that the two proteins are interacting in resting cells. This interaction was further revealed by semiquantification of the spectra after deconvolution (D) (average of six and eight experiments ± S.E.; Student's t test; *, p < 0.05). For this quantification, no correction for bleaching of PI4K-β-ECFP was applied after spectral deconvolution, since this bleaching was too weak to be accurately measured. Cells monotransfected with NCS-1(2A)-EYFP (E) also exhibited a fluorescent peak at 525 nm (excitation 460 nm), which was decreased after illumination. Cells cotransfected with NCS-1(2A)-EYFP and PI4K-β-ECFP (F) displayed a single peak at 495 nm (excitation 430 nm), which was decreased after illumination, indicating that there was no energy transfer between the two chromophores in resting PC12 cells. To assess the specificity of the FRET signal, NCS-1-EYFP was coexpressed with ECFP (G), and PI4K-β-ECFP was coexpressed with EYFP (H). In both cases the fluorescence spectrum (solid line; excitation 430 nm) exhibited a single peak at 495 nm, although the fluorescence of EYFP was detected at 525 nm (excitation 480 nm; dotted and dashed line). A slight bleaching was observed after illumination at 480 nm (dotted line).
FIGURE 5. Kinetics study of PI4K-β and NCS-1 interaction during PC12 cell stimulation revealed by FRET and coimmunoprecipitation. FRET between PI4K-β-ECFP and NCS-1-EYFP in cotransfected cells was quantified by calculating the ratio of EYFP fluorescence intensity on ECFP fluorescence after spectral deconvolution and normalization. Either 80 mM KCl (A) or 30 μM ATP (B) application (bars) induced a reversible increase of the ratio value (filled squares), indicating an additional interaction between PI4K-β-ECFP and NCS-1-EYFP during stimulation. When the cells were cotransfected with PI4K-β-ECFP and NCS-1(2A)-EYFP (open circles), no significant change in the ratio value was observed, indicating no recruitment of NCS-1(2A) by PI4K-β during stimulation (average of five different experiments ± S.E.). The significance of the difference between the two plots was assessed by Student’s t test (*, p < 0.05; **, p < 0.01; ***, p < 0.001). C, 500-μg extracts from resting (R) or 80 mM KCl-stimulated (S) PC12 cells were subjected to immunoprecipitation with a control preimmune serum or with an anti-PI4K-β antibody. 50 μg of extract were resolved by SDS-PAGE (input) and subjected to Western blot analysis using anti-PI4K-β antibody. The immunoprecipitated proteins were also resolved by SDS-PAGE and subjected to Western blot analysis using anti-PI4K-β (IP) and anti-NCS-1 antibodies (Co-IP). Stimulation increased the amount of NCS-1 coprecipitating with PI4K-β. These experiments were repeated three times with different cell preparations. D, PC12 cells expressing NCS-1-GFP or NCS-1(2A)-GFP were processed for PI4K-β immunoprecipitation. The immunoprecipitated proteins were revealed using anti-PI4K-β (IP) and anti-GFP antibodies (NCS-1). No interaction between PI4K-β and NCS-1(2A) was detected. These experiments were repeated with two different cell preparations.
that native NCS-1/PI4K-β complex preexists in PC12 cells and that cell stimulation significantly increased this association.

**Cellular Localization of PI4K-β-NCS-1 Interaction**—In order to determine the precise localization of this additional interaction between PI4K-β and NCS-1, we performed confocal imaging of the FRET signal in resting cells and in stimulated cells. In fixed resting cells overexpressing PI4K-β-ECFP and NCS-1-EYFP, both proteins were observed in the cytoplasm of the cell, displaying a partial colocalization (Fig. 6A). In these cells, a weak but significant FRET signal was observed in the cytoplasm with a FRET efficiency of \(4.3 \pm 0.9\%\) (\(n = 15\)).

Stimulated cells were also fixed immediately after a 1-min KCl application (i.e. before the full response was observed during the kinetics studies) (see Fig. 5), in order to partially avoid the overlap with the translocation phenomenon. In these conditions, the two labeled pro-

![Figure 6. Localization of the FRET signal in resting and stimulated PC12 cells. Confocal micrographs of resting (A) or stimulated (after a 1-min 80 mM KCl application) (B) PC12 cells coexpressing PI4K-β-ECFP and NCS-1-EYFP. The merged images show the colocalization of the two proteins inside the cytoplasm and at the cell periphery (arrows). Pseudocolored FRET efficiency images show a faint FRET signal in resting cells. In stimulated cells, the FRET signal primarily increases in the perinuclear cytoplasm, whereas it seems to decrease at the periphery of the cells (calibration bar, 20 μm). The fluorescence spectrum recorded on the confocal microscope displayed two peaks at 490 and 520 nm, when the preparation was illuminated at 405 nm (continuous line) (C). In cells expressing PI4K-ECFP only a single peak was observed (dotted line) (C). The statistical distribution of the pixel values in FRET signal images shows a single pixel population with low FRET level in resting cells (red columns and dotted line) (D). In stimulated cells (black columns and dashed line) (D), most of the pixels exhibit a high FRET level, but a minor population of low FRET signal pixels is also present. These findings were observed in three distinct cell preparations. Calibration bar, 10 μm.
teins mostly colocalized in the cytoplasm (Fig. 6B) similarly to resting cells and at discrete portions of the cell periphery (arrows). Furthermore, the FRET signal was also detected inside the cytoplasm of stimulated cells (Fig. 6C). The main difference was a significant increase of the FRET efficiency (12.0 ± 1.0%, n = 12) mostly in the perinuclear region and at the cell periphery in complete agreement with our microspectrofluorimetric measurement (see Fig. 5). In these preparations, however, a statistical analysis of the pixel values revealed a minor pixel population (about 20%) not responding to the stimulation (Fig. 6D). Most of the pixels displaying a clear increase in the FRET signal were in cellular compartments where the colocalization of the two proteins was important (Fig. 6B). In addition, there was a moderate change in the FRET signal intensity near the membrane of the stimulated cells but a clear decrease of the signal in the cytoplasm at the periphery of the cell (Fig. 6B). These observations suggest that stimulation induces the rapid translocation of preexisting PI4K-β-NCS-1 complexes toward the plasma membrane and the slow recruitment of additional complexes in the perinuclear zone, possibly in the trans-Golgi apparatus.

Functional Implication of the NCS-1-PI4K-β Interaction—We verified that overexpressed PI4K-β-ECFP and NCS-1-EYFP were able to modulate the secretory function of living PC12 cells. The functional implication of NCS-1 myristoylation was also further assessed by performing secretion measurements in cells overexpressing native NCS-1 or mutated NCS-1 using the classical GH assay (Fig. 7). In control experiments, we detected no significant difference in the total amount of GH expressed in control cells and in PI4K-β-ECFP- and NCS-1-EYFP-transfected cells (not shown). Additionally, NCS-1 and PI4K-β expres-
sion did not affect the distribution of GH in secretory granules (not shown). Whereas the basal level of secretion was not significantly modified by overexpression of either native NCS-1-EYFP or mutated NCS-1-EYFP, we observed a consistent slight increase of KCl- or ATP-induced secretion in cells overexpressing NCS-1-EYFP. This effect was abolished in cells overexpressing NCS-1-EYFP. A modest but significant increase in GH secretion was also observed in cells overexpressing mutated NCS-1-EYFP. Co-expression of NCS-1-EYFP with PI4K-β-ECFP induced a strong increase of secretion (about 40%) when the cells were stimulated by KCl or ATP applications. The synergistic effect of NCS-1 and PI4K-β on the evoked secretion is to be compared with the increased interaction as revealed by FRET measurements. In addition, this secretion stimulation was completely inhibited in cells overexpressing mutated NCS-1-(2A)-EYFP together with PI4K-β-ECFP, suggesting that, like the physical interaction detected by FRET, the functional interaction of NCS-1 with PI4K-β strictly requires myristoylation of NCS-1.

In order to bypass Ca\(^{2+}\) channels, we permeabilized PC12 cells with digitonin, which allows direct control of the intracellular Ca\(^{2+}\) concentration and allows us to study the effect of NCS-1 overexpression independently of its effect on channel activity. As reported in Fig. 8C, overexpression of NCS-1 significantly increased the secretory activity in response to 20 \(\mu\)M Ca\(^{2+}\). This effect was very similar to the stimulatory effect observed for KCl- or ATP-stimulated cells, which is in agreement for an action of NCS-1 on exocytosis distal to Ca\(^{2+}\) entry.

To directly address the role of NCS-1-PI4K-β interaction in PC12 cell exocytosis, we selectively inhibited native levels of PI4K-β by taking advantage of the possibilities offered by the RNA interference process. We engineered a plasmid to express both GH and siRNAs targeted against the sequence of PI4K-β. Transient expression of the PI4K-β silencer in PC12 cells selectively reduced the level of PI4K-β but did not affect the expression of actin or tubulin (not shown), as assessed by immunodetection on nitrocellulose sheets (Fig. 8A). Densitometry scanning of the films from three independent experiments revealed that the total level of PI4K-β in the cell culture was decreased by 52 ± 6% (Fig. 8B). Taking into account that 60% of the cells were transfected, this result indicates that on average, the level of endogenous PI4K-β was reduced by 86% in cells expressing GH and the active PI4K-β siRNA in agreement with our recent results obtained for
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PI4K-β extinction in INS-1E insulin-secreting cells (25). The specificity of the siRNA was verified by using an inactive sequence and the active sequence mutated for the two first bases (Fig. 8, A and B). The involvement of PI4K-β in exocytosis was then estimated by measuring basal and Ca²⁺-evoked secretion of GH. Expression of PI4K-β siRNAs only moderately reduced the expression level of GH (∼15%; see Fig. 8C) and did not affect the distribution of GH in secretory granules (not shown). Selective knockdown of PI4K-β by siRNA also did not modify basal secretion, but it resulted in a significant decrease (−42% inhibition) in the amount of GH released in response to stimulation by 20 μM Ca²⁺ (Fig. 8D), which is consistent with the ∼60% inhibition of insulin secretion observed in INS-1E cells expressing the active PI4K-β siRNA (25). More importantly, PI4K-β extinction completely prevented the stimulatory effect of NCS-1 on exocytosis (Fig. 8C). Overall, these findings are consistent with the idea that the NCS-1-PI4K-β complex plays a positive role in the exocytotic pathway of large dense core granules.

DISCUSSION

Distribution of NCS-1 and PI4K-β in PC12 Cells—Endogenous NCS-1 and PI4K-β were mostly found in the cytoplasm of resting PC12 cells. Both proteins were not evenly distributed through the cytoplasm but appeared partially enriched in the perinuclear region and near the plasma membrane. These observations are in agreement with previous studies showing either by immunocytochemistry (1) or by subcellular fractionation and subsequent biochemical analysis (12, 13) that both proteins were essentially present in the Golgi apparatus and the cytoplasm and/or associated with vesicles. However, in addition to other reports (13), we also show here that a fraction of either protein is present at the cellular periphery, beneath discrete portions of the plasma membrane in resting cells. Are NCS-1 and PI4K-β directly associated to the plasma membrane? In resting conditions, both biochemical and immunofluorescence experiments have shown that around 10–15% of NCS-1 and PI4K-β colocalize with the plasma membrane marker SNAP-25.

Recruitment and Interaction of NCS-1 and PI4K-β—A significant recruitment of NCS-1 and PI4K-β toward the cell periphery occurred when the cells were stimulated either by KCl-induced depolarization or by ATP application. These relocalizations appear to occur largely at the expense of the cytoplasmic pool of NCS-1 and PI4K-β.

Interestingly, our imaging experiments also showed for the first time that this redistribution was reversed shortly after stimulus withdrawal and was highly correlated with [Ca²⁺], changes. However, we observed a significant difference in the dissociation kinetics between the two proteins: for PI4K-β, the kinetics was biexponential, similar to that of [Ca²⁺], whereas the kinetics of NCS-1 was monoeponential and rapid. These observations strongly suggest that Ca²⁺ differentially regulates the backward movement of the two proteins.

We observed that a fraction of NCS-1 and PI4K-β interact in resting living cells, hence in the presence of low Ca²⁺ concentration. This result is in agreement with a previous report (13), which showed that NCS-1 and PI4K-β co-immunoprecipitated in extracts from resting PC12 cells. Additionally, we also observed that during cell stimulation, there was an increase in the interaction between NCS-1 and PI4K-β as revealed by FRET and co-immunoprecipitation. The change in FRET intensity might be explained by a Ca²⁺-induced conformational change of the proteins, which would be expected for the calcium sensor NCS-1. Alternatively, this additional FRET signal may reflect a Ca²⁺-triggered recruitment of NCS-1 and/or PI4K-β in the same subcellular compartment, together with the formation of an additional complex between NCS-1 and PI4K-β. Since this increase of the FRET signal is slower than the kinetics of the [Ca²⁺], response during stimulation and coincides with a cellular compartment where the colocalization is maximal, we would favor the latter hypothesis. This agrees with an increase of the immunoprecipitated complex, NCS-1-PI4K-β. This additional interaction was transient, and the backward translocation kinetics was different for the two proteins. This also reveals a partial dissociation of the two proteins when [Ca²⁺], returned to basal level.

Functional Aspect of the Interaction of NCS-1 and PI4K-β—An interesting insight into the physiological role of NCS-1 has come from the work of Hendricks et al. (9), demonstrating that the yeast homologue of NCS-1 can associate with and up-regulate the activity of PI(k)l homologous to the mammalian PI4K-β type III enzyme. It was further confirmed that PI4K-β type III and NCS-1 interact in vitro (11, 12). Since it has been largely documented that PIP₂, the final product of phosphoinositide 4- and phosphoinositide 5-kinase, plays an important function in various aspects of vesicular trafficking, including regulated exocytosis of secretory granules, an interesting hypothesis is that NCS-1 could be the calcium sensor that adjusts the enzymatic activity of PI4K-β to [Ca²⁺]. Indeed, there are several proteins implicated in exocytosis that specifically bind to PIP₂ like the calcium sensor synaptotagmin and Rabphilin 3 (26, 27). In addition, we have recently documented that the lipid-modifying enzyme phospholipase D1, plays an important role in a late phase of the exocytotic process and also requires binding to PIP₂ at the plasma membrane for activity (28). In fact a GFP-fused pleckstrin homology domain, which specifically binds PIP₂, revealed that the PIP₂ pool involved in exocytosis is predominantly generated at the active exocytotic site in chromaffin cells (29), which is in agreement with our findings. An interesting speculation is that PI4K-β activity generates phosphoinositol 4-phosphate, which will be the substrate for the cytosolic phosphoinositide 5-kinase enzyme. Therefore, PI4K-β may regulate temporally and spatially the formation of PIP₂-containing raftlike structures at the plasma membrane, which would delineate exocytotic sites.

Our results demonstrate for the first time in living cells that NCS-1 myristoylation is necessary for the formation of NCS-1-PI4K-β complex and that this complex is also required for the stimulation of evoked secretion. Using silencing experiments, we also demonstrated directly that the stimulatory effect of NCS-1 on exocytosis required endogenous PI4K-β. Thus, although the stimulatory action of NCS-1 on PI4K-β activity in vitro and in cells has been well documented (10–13), our present result represents the first direct demonstration of the functional link between NCS-1, PI4K-β, and regulated exocytosis. These findings also establish the specificity of the NCS-1-PI4K-β interaction detected by FRET and confirms the necessity of NCS-1 insertion into a membrane compartment to achieve a functional interaction with PI4K-β in PC12 cells. An initial model postulated that the interaction process was triggered by [Ca²⁺], (14, 15), but this was later challenged by O’Callaghan et al. (17), who demonstrated in vitro that the myristoyl residue of NCS-1 was able to insert into lipid membranes without Ca²⁺. From the present study, we can conclude that in living PC12 cells both phenomena coexist: an interaction in the presence of low Ca²⁺ concentrations and a subsequent Ca²⁺-triggered additional interaction.

What is the physiological significance of such a dual regulation? Since we did not measure differences in basal secretion level between control cells and cells overexpressing NCS-1 and PI4K-β, the protein complex is probably not active in the absence of Ca²⁺ or at least not directly involved in terms of secretion. From our kinetics studies, we can distinguish the slow formation of the NCS-1-PI4K-β complex from the rapid recruitment of the proteins, which is correlated to [Ca²⁺], changes. Our results also show that the NCS-1-PI4K-β complex exists prior to the
translocation. We thus favor the hypothesis that the formation of the complex at the cell periphery occurs at or near exocytotic sites. Indeed, some secretory granules are predocked to the plasma membrane, as revealed by the presence of SNARE complexes in resting cells (30). Upon cell stimulation, there is a massive and rapid translocation of the secretory granules toward the exocytotic spots, which correlates with a drain of NCS-1:PI4K-β complex from some part of the cytoplasmic compartment. In agreement with our functional analysis is the observation of a significant increase in FRET signal at the plasma membrane after cell depolarization. Stimulation also triggers a slow additional reaction of complexes could be involved in the generation of a significant increase in FRET signal at the plasma membrane within the trans-Golgi network. The first set of complexes may regulate the release competence of secretory granules from PC12 cells (13) (this study), pancreatic β cells (31), neurons (32), and mast cells (33) probably at the level of priming or fusion reactions. The second set of complexes could be involved in the generation of cargo, such as large dense core secretory granules. Indeed, it has been shown recently that NCS-1 and the small GTPase ARF1 display an antagonistic action toward PI4K-β (35). NCS-1 was shown to modulate the effect of ARF1 on the Golgi apparatus, and a functional cross-talk between Ca²⁺-dependent and ADP-ribosylation factor-dependent pathways in the trans-Golgi network to plasma membrane traffic was suggested (34). Our results indicating for the first time that a large part of PI4K-β and NCS-1 interaction occurs in the perinuclear region are in complete agreement with this study (34). In addition, the data indicating a small but significant reduction in GH synthesis (Fig. 8C) in cells expressing active siRNA toward PI4K-β also favor this hypothesis. Future study will now have to directly establish the role of phospholipid metabolism in large dense core secretory granule biogenesis.

Acknowledgments—We thank Dr. A. F. Parlow and the NIDDK, National Institutes of Health, National Hormone and Pituitary Program for generously providing polyclonal anti-GH antibodies, Dr. M. H. Metz-Boutigue for the anti-CGA antibody, and Drs. R. Regazzi and L. Wassell for the PI4K-β siRNA sequence. We are grateful to Dr. M. F. Bader for helpful comments on the manuscript and to T. Thaouly for technical assistance. We acknowledge the confocal microscopy facilities of Plateforme Imagerie In vitro of IFR37.

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JUNE 30, 2006•VOLUME 281•NUMBER 26
JOURNAL OF BIOLOGICAL CHEMISTRY 18111