Phosphatidylinositol 4-OH Kinase Is a Downstream Target of Neuronal Calcium Sensor-1 in Enhancing Exocytosis in Neuroendocrine Cells*

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Neuronal calcium sensor-1 (NCS-1), the mammalian orthologue of frequenin, belongs to a family of EF-hand-containing Ca\(^{2+}\) sensors. NCS-1/frequenin has been shown to enhance synaptic transmission in PC12 cells and Drosophila and Xenopus, respectively. However, the precise molecular mechanism for the enhancement of exocytosis is largely unknown. In PC12 cells, NCS-1 potentiated exocytosis evoked by ATP, an agonist to exocytosis is largely unknown. Most family members are solely expressed in neuronal and neuroendocrine cells, but a frequenin homologue has been identified in yeast, suggesting a more general role for this protein in mediating Ca\(^{2+}\) responses.

All members of the NCS family share four EF-hand motifs and a myristoylated N terminus. For NCS-1, three of the four EF-hands are functional Ca\(^{2+}\)-binding motifs, whereas the EF-hand closest to the N terminus (EF1) is non-functional (2). Biochemical and structural analysis of recoverin have led to the calcium/myristoyl switch model in which Ca\(^{2+}\) binding to the NCS proteins may trigger their translocation from the cytosol to intracellular membranes (3, 4). This model suggests that Ca\(^{2+}\) binding to the NCS proteins induces large conformational changes, resulting in the exposure of the myristoyl group believed to allow membrane attachment. In addition, the movement of the myristoyl group is thought to expose a hydrophobic pocket within the protein that could then interact with target proteins (4). However, recent studies suggest that the calcium/myristoyl switch is not a general feature of all members of this family, because the localization of NCS-1 was found to be independent of Ca\(^{2+}\), indicating that the myristoyl group may be freely accessible in the absence of Ca\(^{2+}\) binding to NCS-1 (5).

Overexpression of frequenin in Drosophila facilitates evoked neurotransmission at the neuromuscular junction (6), and injection of frequenin into Xenopus spinal neurons enhances both spontaneous and evoked neurotransmission (7). NCS-1 is also present in adrenal chromaffin and PC12 neuroendocrine cells, where its overexpression has been shown to increase Ca\(^{2+}\)-regulated exocytosis from dense-core granules (8), analogous to the effect reported for Drosophila frequenin in enhancing synaptic vesicle exocytosis. However, the mechanism(s) by which NCS-1/frequenin regulates vesicular release is not known.

Neuronal calcium sensor-1 (NCS-1), the mammalian homologue of frequenin, belongs to the EF-hand family of Ca\(^{2+}\)-binding proteins (1, 2), which includes recoverin/S-modulin, visinin, visinin-like proteins, neurocalcin, and hippocalcin. Some members of this family, such as recoverin/S-modulin and visinin, are expressed only in photoreceptor cells and are thought to function in the control of visual transduction pathways, whereas the functional roles of the other members are largely unknown. Most family members are solely expressed in neuronal and neuroendocrine cells, but a frequenin homologue has been identified in yeast, suggesting a more general role for this protein in mediating Ca\(^{2+}\) responses.

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‡ The abbreviations used are: NCS, neuronal calcium sensor; PI4K, phosphatidylinositol 4-OH kinase (PI4K). Plasma membrane phosphatidylinositol 4,5-bisphosphate pools were increased upon NCS-1 transfection as visualized using a phospholipase C-δ pleckstrin homology domain-green fluorescent protein construct. NCS-1-transfected cell extracts displayed increased phosphatidylinositol-4-phosphate biosynthesis, indicating an increase in PI4K activity. Mutations in NCS-1 equivalent to those that abolish the interaction of recoverin, another EF-hand-containing Ca\(^{2+}\) sensor, with its downstream target rhodopsin kinase, lost their ability to enhance exocytosis. Taken together, the present data indicate that NCS-1 modulates the activity of PI4K, leading to increased levels of phosphoinositides and concomitant enhancement of exocytosis.

Neuronal calcium sensor-1 (NCS-1), the mammalian orthologue of frequenin, belongs to a family of EF-hand-containing Ca\(^{2+}\) sensors. NCS-1/frequenin has been shown to enhance synaptic transmission in PC12 cells and Drosophila and Xenopus, respectively. However, the precise molecular mechanism for the enhancement of exocytosis is largely unknown. In PC12 cells, NCS-1 potentiated exocytosis evoked by ATP, an agonist to exocytosis is largely unknown. Most family members are solely expressed in neuronal and neuroendocrine cells, but a frequenin homologue has been identified in yeast, suggesting a more general role for this protein in mediating Ca\(^{2+}\) responses.

All members of the NCS family share four EF-hand motifs and a myristoylated N terminus. For NCS-1, three of the four EF-hands are functional Ca\(^{2+}\)-binding motifs, whereas the EF-hand closest to the N terminus (EF1) is non-functional (2). Biochemical and structural analysis of recoverin have led to the calcium/myristoyl switch model in which Ca\(^{2+}\) binding to the NCS proteins may trigger their translocation from the cytosol to intracellular membranes (3, 4). This model suggests that Ca\(^{2+}\) binding to the NCS proteins induces large conformational changes, resulting in the exposure of the myristoyl group believed to allow membrane attachment. In addition, the movement of the myristoyl group is thought to expose a hydrophobic pocket within the protein that could then interact with target proteins (4). However, recent studies suggest that the calcium/myristoyl switch is not a general feature of all members of this family, because the localization of NCS-1 was found to be independent of Ca\(^{2+}\), indicating that the myristoyl group may be freely accessible in the absence of Ca\(^{2+}\) binding to NCS-1 (5).

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A wide array of binding partners has been identified for NCS-1/frequenin, only some of which may be of physiological relevance. NCS-1/frequenin has been shown to activate membrane-bound guanylate cyclase and to inhibit rhodopsin kinase (6, 9). However, these in vitro effects on photoreceptor proteins are unlikely to be relevant to the function of NCS-1/frequenin in vivo in neurons and neuroendocrine cells. NCS-1 also seems to activate various calmodulin targets, such as cyclic nucleotide phosphodiesterase, calcineurin, and nitric-oxide synthase (10, 11), and to modulate a variety of ion channels such as voltage-gated Ca\(^{2+}\) channels (12–15) and A-type K\(^{+}\) channels (16). Despite these interactions, the binding partners for NCS-1 in vivo are largely unknown.
are not known for certain, and thus the molecular downstream targets mediating NCS-1 function remain to be determined.

Recently, yeast freqeenin was demonstrated to genetically and biochemically interact with, and regulate the activity of, the yeast phosphatidylinositol 4-OH kinase (PI4K) Pik1 (17), which itself has been shown to be essential for Golgi-to-cell surface vesicular trafficking (18, 19). Similarly, the mammalian Pik1 homologue PI4Kβ (20) has been shown to interact with NCS-1 in vitro and if immunoprecipitated upon co-expression in non-neuronal cells (21, 22), suggesting that PI4Kβ may be an evolutionarily conserved and functionally important downstream target of NCS-1/freqeenin.

Inositol phospholipids have recently emerged as important regulators of exo- and endocytosis (23, 24). Regulated exocytosis of dense core vesicles has been shown to require a phospholipase C (PLC) activated by G-protein-coupled receptor agonists. However, in both neuronal and non-neuronal cells, PtdIns(4,5)P2 has been shown to play an important role in exocytosis independent from its PLC-mediated cleavage (29, 30), and PtdIns(4,5)P2 is likely to act directly on a specific target (31).

In the present study, we provide evidence that NCS-1 enhances secretion from PC12 cells through regulating the activity of PI4K. Overexpression of NCS-1, but not of its myristoylation mutant, led to an enhancement of ATP- and ionomycin-evoked release. NCS-1 expression also decreased the sensitivity of the cells to phenylarsine oxide (PAO), an inhibitor of PI4K, and increased the levels of PtdIns(4,5)P2 at the plasma membrane. Finally, three mutations in NCS-1 equivalent to those that interfere with the binding of recoverin to rhodopsin kinase had lost their enhancing effect on exocytosis, suggesting that NCS-1 acts through activating PI4K, leading to enhanced PtdIns(4,5)P2 levels and a downstream enhancement of regulated secretion.

EXPERIMENTAL PROCEDURES

Construction of Expression Vectors—The NCS-1 construct was generated as described previously (32). The PI4Kβ, PI4Kδ, PI4Kβδ (D656A), and PI4KδN terminus-GFP constructs were kindly provided by Rachel Meyers and have been described previously (33). The INP51-GFP and PH-EGFP/H/R40L)-EGFP constructs were donated by John York and Tamas Balla, respectively.

PCMV5-hGH was generated by subcloning the coding region of hGH, amplified by PCR and incorporating 5′ XbaI and 3′ sites, into the XbaI site of the pCMV5 vector. The identity of all constructs was verified using automated DNA sequencing.

PC12 Cell Culture, Transfection, and Secretion Experiments—Cell culture, transfection, and secretion experiments were performed as described (34) with some modifications. PC12 cells (Riken Cell Bank) were cultured on 100-mm dishes coated with collagen (rat tail collagen type I; BD Biosciences). Cells were grown at 37 °C in 5% CO2 in full medium (RPMI 1640 with heat-inactivated 10% horse serum and 5% fetal bovine serum, penicillin (50 units/ml), and streptomycin (50 units/ml) on 100-mm dishes. The next day (90% confluency), secretion experiments were performed with all test and control conditions carried out on the same pool of transfected cells. Controls were treated with 0.5 ml of physiological saline solution (PSS; 145 mM NaCl, 5.6 mM KCl, 2.2 mM CaCl2, 0.5 mM MgCl2, 5.6 mM glucose, 15 mM Hepes-NaOH, pH 7.4). ATP-evoked secretion was stimulated by a 10-min incubation with ATP buffer (PSS containing 300 μM ATP) and 5 mM CaCl2. ATP was preincubated with high K+ saline solution (PSS containing 95 mM NaCl and 56 mM KC1), and ionomycin-triggered secretion was stimulated by a 10-min incubation with ionomycin (5 μM ionomycin (Sigma) in PSS, final MeSO concentration 0.5% (v/v)). Cells were incubated with a-latrotoxin (final 1.5 μM) in PSS for 10 min. At the end of the experiment, dishes were transferred to ice, and the supernatant was removed and centrifuged for 10 min in an Eppendorf centrifuge. hGH in the supernatant from this centrifugation was taken as secreted hGH. The cells in the dishes were resuspended in 0.5 ml of ice-cold phosphate-buffered saline containing 1 mM EDTA and added to the pellet of the Eppendorf centrifugation of the medium. Cells were then lysed by five freeze-thaw cycles in a dry ice/ethanol bath and a 37 °C heating block, and insoluble material was pelleted in an Eppendorf centrifuge. hGH in the supernatant from this centrifugation was taken as the cellular hGH that was not secreted. hGH levels in the various samples were measured using an enzyme-linked immunosorbent assay kit (Roche Molecular Biochemicals) according to the manufacturer’s instructions. All experiments were performed in duplicates or triplicates, and the average percent of total hGH released was calculated. Statistical analyses were performed with the paired Student’s t test.

Western Blot Analysis of Overexpressed Proteins in PC12 and COS-7 Cells—PC12 cells were plated onto collagen-coated 6-well tissue culture plates at 80% confluency as described above. COS-7 cells were grown at 37 °C in 5% CO2 in COS cell medium (Dulbecco’s medium with 10% fetal bovine serum, penicillin (50 units/ml), and streptomycin (50 units/ml)) on 100-mm dishes. The next day (90–95% confluency), cells were transfected with the appropriate vector using 2.4 μg of vector/well of a 6-well plate for PC12 cells and 10 μg of vector/100-mm dish for COS-7 cells, respectively, using LipofectAMINE 2000 (Invitrogen) as described above. One day later, cells were lysed using 500 μl of hot 1% SDS, and cell lysates were centrifuged at 12,000 χ g for 10 min. Protein concentration in the supernatant was determined by a BCA assay (Pierce). Equal amounts of total protein were separated by SDS-PAGE and transferred to Hybond-N membranes (Amersham Biosciences). Membranes were blocked with 2% non-fat dry milk in TTBS (TBST containing 20, 150 mM NaCl, 0.1% (w/v) Tween 20, 50 units/ml), pH 7.5) for 1 h at room temperature and hybridized with primary antibody (either anti-NCS-1 rabbit polyclonal or anti-PI4Kβ rabbit polyclonal, respectively, at 1:1000 dilution) overnight at 4 °C. After washing in TTBS, blots were incubated with a horseradish peroxidase-tagged anti-rabbit antibody (DARCO A/S) at 1:2000 dilution for 1 h at room temperature, washed, and developed using an enhanced chemiluminescence system (Amersham Biosciences).

Inhibition of PI4K Activity—Cells were co-transfected with either pcMV5-hGH and pcDNA3.1 or with pcMV5-hGH and pcDNA3.1-NCS-1, as described above. Cells were incubated in RPMI 1640 medium containing various concentrations of phenylarsine oxide (Sigma) (final concentrations maintained in all cultures) at 37 °C in 5% CO2 overnight at 4 °C. Cells—membrane fractionation was performed as described above.

Immunofluorescence—To visualize localization of endogenous NCS-1 and PI4Kβ, non-transfected cells were plated onto coverslips coated with poly-L-lysine (coated for 1 h with 50 μg/ml poly-L-lysine, M, 80K; Sigma) and allowed to grow for 3 days at 37 °C in 5% CO2. Cells were permeabilized in 0.5% Triton X-100 in PBS for 30 min and preincubated in blocking buffer (10% goat serum (Vector Laboratories) in 0.1% (w/v) Triton X-100 in PBS containing 0.2% (w/v) paraformaldehyde diluted in blocking buffer) for 1 h at room temperature. Cells were washed in 0.5% Triton X-100 in PBS for 3 min and incubated with secondary antibodies for 1 h at room temperature.

To determine the amount of overexpression of NCS-1, as well as the
amount of co-expression of NCS-1 and hGH. 50% of cells were processed for secretion assays, whereas the remainder were replated onto poly-l-lysine-coated coverslips and fixed and processed for immunocytochemistry 2 days after replating. The amount of overexpressed protein was qualitatively assessed using antibody dilutions that barely visualized endogenous protein levels but easily detected overexpressed proteins. The amount of co-expression was calculated by counting the number of cells expressing both hGH and the protein of interest, as compared with cells expressing either one or the other protein only. Transfection efficiencies were calculated by counting the number of cells expressing either proteins, and 700–1200 cells were counted for each construct. Transfection efficiencies regularly amounted to around 15%, and all NCS-1 constructs reached co-transfection efficiencies of ≥90%.

The following antibodies were used: anti-NCS-1 rabbit polyclonal (44162) (35) or chicken polyclonal (22), anti-PI4Kβ rabbit polyclonal (Upstate Biotechnology), anti-synaptobrevin/VAMP2 mouse monoclonal (Cl 69.1; Synaptic Systems), anti-syntaxin 1 mouse monoclonal (HPC-1; Sigma), and anti-hGH rabbit polyclonal (National Hormone and Pdk Program, NIDDK, National Institutes of Health). The specificities of the polyclonal anti-NCS-1 and anti-PI4Kβ antibodies have been described previously (15, 33). Secondary antibodies included horse anti-mouse IgG conjugated to fluorescein isothiocyanate or Texas red (TR), goat anti-rabbit IgG conjugated to fluorescein isothiocyanate or TR (all from Vector Laboratories) and rabbit anti-rabbit IgG conjugated to fluorescein isothiocyanate (Sigma). Cells were either examined on a confocal microscope (Leica TCS NT) under a ×40 oil-immersion objective, and data were acquired using Leica software or on an upright microscope (Leica) under a ×40 or ×100 oil-immersion objective, and data were acquired using IPLab.

**PH Domain Imaging and Analysis**—Cells were co-transfected with NCS-1 or NCS-1(22A) and either phospholipase C-δ PH-domain-GFP construct (PH-EGFP) or PH(R40L)-EGFP, a mutant unable to bind PtdIns(4,5)P₂ (36). Cells were either visualized live or after fixation and permeabilization as described above, 2 days after transfection, with identical results. For experiments measuring PH-EGFP intensities upon PAO treatment, cells were fixed but not permeabilized. To quantify intensities, rectangles of 80 × 110 pixels centered on each GFP-punctate cell were collected from each random field acquired using a ×40 oil-immersion objective. Pixel intensities within each rectangle were calculated in IPLab, and a total of 100 cells were counted for each condition. Cells with the highest PH-EGFP expression levels were excluded, as they often gained a rounded appearance and formed membrane blebs (37). Experiments were done three times, and the data were analyzed using the paired Student’s t test.

**Electroporation of Cells, Endogenous Lipid Phosphorylation, and Thin Layer Chromatography**—PC12 cells were resuspended in RPMI 1640 medium (3 × 10⁶ cells/ml). Cells (400 μl) were incubated with 15 μg of pcDNA3.1-NCS-1, 15 μg of pcDNA3.1, or with 15 μg of pCMV5-GFP for 3 min and electroporated (330 V, 20 ms) using a ECM 830 Square Wave Electroporator (BTX; Genetronics Inc., San Diego, CA). Electroporated cells were immediately transferred into 10 ml of full medium, washed once, and counted. Under the indicated electroporation conditions, cell survival usually amounted to around 10–15%, and electroporation efficiency, as measured by counting the fraction of cells expressing GFP 2 days after electroporation, amounted to around 50%. Cells were plated onto collagen-coated 100-mm dishes at 5 × 10⁶ cells/dish. Two days later (95% confluency), attached cells were harvested, washed into PBS, and counted, and 0.5 × 10⁶ cells were pelleted in Eppendorf tubes. Pellets were quick-frozen in liquid N₂ until further use.

Cells were resuspended in 100 μl of kinase buffer (20 mM Hepes/NaOH, pH 7.5, 100 mM NaCl, 10 mM MgCl₂) and disrupted by passing five times through a 17-gauge needle syringe. The aliquot of the prepared lysate was used directly in an assay, or the resulting lysate was further fractionated by centrifugation at 10,000 × g for 15 min at 4 °C. The supernatant of this centrifugation was taken as cytosol fraction, and the pellet was resuspended in kinase buffer and taken as membrane fraction. PI4K activity was measured as described previously (61, 62). Briefly, to initiate phospholipid biosynthesis, cell lysate and membrane fractions were diluted with kinase buffer to 45 μl, and 5 μl of 0.5 mM ATP containing 10 μCi of [γ-³²P]ATP (3000–6000 Ci/mmol; PerkinElmer Life Sciences) was added. The reaction was incubated for 10 min at room temperature and stopped by adding 60 μl of 1 x HCl. A two-phase mixture was induced, and the phospholipid radiolabeled lipids were extracted by adding 160 μl of chloroform:methanol, 1:1 (v/v). The extracted products were analyzed by thin layer chromatography (TLC) (silica gel 60 thin layer plates, using chloroform/methanol/15% ammonium hydroxide/water (90:90:7:22) (v/v/v/v) as the solvent system). Radioactive bands were detected on x-ray film.
are transiently co-transfected into PC12 cells. In transfected cells, hGH is packaged into vesicles of the regulated secretory pathway and serves as a reporter for exocytosis as a function of stimulation. A high probability of co-transfection of two distinct plasmids into the same cells makes it possible to investigate the effect of the protein of interest on hGH secretion. A change in hGH secretion upon co-transfection of a particular protein is taken as evidence for an involvement of the protein in exocytosis. This assay has been successfully used to implicate a variety of proteins in exocytosis (34, 44–46) and thus serves as a useful model system to study the role of NCS-1 and PI4Kβ in secretion.

Extracellular ATP is thought to evoke secretion from PC12 cells by binding to two purinergic receptors: a P2Y receptor, which leads to the activation of PLC and concomitant inositol 1,4,5-trisphosphate-mediated release of Ca^{2+} from intracellular stores, and a P2X non-selective cation channel, which leads to Ca^{2+} entry across the plasma membrane (47, 48). ATP caused robust hGH secretion from PC12 cells co-transfected with control vector, which was enhanced when cells were co-transfected with NCS-1 (Fig. 2A), as reported previously (8). In the absence of secretagogue, the amount of hGH in the PC12 cell medium (%10%) did not increase upon NCS-1 transfection, indicating that NCS-1 does not induce a significant amount of constitutive secretion of hGH (Fig. 2A). Expression of NCS-1 did not alter the expression levels of hGH (Fig. 2B), indicating that the enhancement of exocytosis observed with NCS-1 is not because of a change in the relative amounts of expressed hGH. NCS-1 enhanced ATP-stimulated secretion by 31 ± 8% (n = 9, p < 0.01; see Fig. 2C). To test the functional relevance of the myristoyl group in NCS-1, cells were co-transfected with hGH and NCS-1(E22A), a point mutation that renders the myristoylation consensus sequence non-functional. This mutant was unable to enhance ATP-evoked exocytosis (3.6 ± 9.5% enhancement; n = 6; see Fig. 2C). In addition, a mutant in EF-hand EF3 (NCS-1(E120Q)) that has been shown to have impaired Ca^{2+}-binding activity (12) still enhanced ATP-evoked secretion, albeit to a lesser extent as compared with wild-type NCS-1 (15.8 ± 1.9% enhancement; n = 5, p < 0.01; see Fig. 2C).

Double immunocytochemistry was performed on cells processed in parallel for secretion assays, using an anti-hGH antibody and an anti-NCS-1 antibody at a dilution that barely visualized endogenous protein but easily recognized overexpressed protein. For both mutant and wild-type NCS-1 constructs, transfection efficiencies were around 15%, and co-transfection efficiencies were comparable and ≥90% (data not shown). In addition, the level of overexpression of the different NCS-1 constructs was analyzed using both Western blotting and immunocytochemistry (Fig. 3, A and B). As assessed by immunocytochemistry and antibody concentrations that barely visualized endogenous protein, all three NCS-1 constructs were heavily overexpressed as compared with endogenous NCS-1 (8–10-fold), and no partial degradation of mutant NCS-1 constructs was detected by Western blotting (Fig. 3, A and B), indicating that the relative or total lack of an effect of the NCS-1 mutants on ATP-evoked secretion is not because of a difference in co-transfection efficiencies, expression levels, or partial degradation of expressed proteins. Overexpressed NCS-1 was found partially cytosolic and partially bound to the plasma membrane (Fig. 3, A and B). The myristoylation mutant NCS-1(22A) was largely cytosolic (Fig. 3B), supporting the notion that the myristoyl anchor is required for membrane attachment. The Ca^{2+}-binding mutant NCS-1(E120Q) was membrane-associated like wild-type NCS-1 (Fig. 3B), in agreement with the observation that the localization of NCS-1 is independent of Ca^{2+} (5).

Next, we tested whether co-expression of PI4Kβ, or mutants thereof, affect ATP-evoked hGH secretion. Neither wild-type PI4Kβ, nor PI4Kβ(D656A), a catalytically inactive mutant (33), nor a GFP-tagged N-terminal fragment (PI4K-N-term.) changed ATP-evoked secretion, as compared with empty control vector (Fig. 2C). Immunocytochemistry with an anti-PI4Kβ antibody (data not shown), as well as Western blot analysis, revealed that none of the three PI4Kβ constructs were significantly expressed above endogenous protein levels, even though they could be overexpressed in COS-7 cells (Fig. 3C). Although not detectable above endogenous PI4Kβ levels, expression of the GFP-tagged N-terminal fragment (PI4K-N-term.) could be demonstrated (Fig. 3D). Finally, decreasing PtdIns(4,5)P_2 levels presumably at the plasma membrane by co-expression of a GFP-tagged PtdIns(4,5)P_2-phosphatase (INP51) predominantly plasma membrane-targeted by an N-terminal myristoy-
limiting concentrations or because of a lack of efficient overex-
expression of NCS-1 constructs in NGF-differentiated cells was as-
alyzed in live cells. Scale bar, 10 μm.

Differential Effect of NCS-1 on hGH Secretion Evoked by Different Secretagogues—As another means to address whether NCS-1 acts to enhance secretion by activating PI4Kα, we tested its effects on Ca^{2+}-dependent secretion stimulated by secreta-
gogues with distinct mechanisms of action. KCl at high concen-
trations induces Ca^{2+} influx by membrane depolarization, ionomycin acts as a Ca^{2+} ionophore, leading to global increases in [Ca^2+]i, and α-latrotoxin triggers exocytosis by an unknown mechanism. Both KCl and ionomycin evoke secretion in a PLC-independent manner in PC12 cells (49). Although ATP-evoked hGH secretion was enhanced upon NCS-1 transfection (23.8 ± 2.7% increase, n = 5, p < 0.005; see Fig. 4, A and D), there was no effect on secretion when cells were stimulated with KCl (3.2 ± 4.4% increase, n = 5; see Fig. 4B) or α-latrotoxin (data not shown). There was a slight, variable NCS-1-dependent stimulation of hGH secretion induced by ionomycin (10 ± 6.7%; p < 0.25, n = 5; see Fig. 4, C and D).

If NCS-1 enhances secretion through activating PI4Kα and concomitantly increasing PtdIns(4,5)P_2 levels, the enhance-
ment of ionomycin-stimulated release suggests that PtdIns-
(4,5)P_2 levels may be directly relevant for exocytosis down-
stream from Ca^{2+} entry in a manner independent of PLC. In that case, it seems unclear why KCl-evoked release is not equally potentiated upon the expression of NCS-1. However, overexpressed NCS-1 was found enriched at the plasma mem-
brane (Fig. 3) and thus may, at least in part, buffer depolar-
ization-induced Ca^{2+} influx, which may mask its effect in enhancing exocytosis. Indeed, upon overexpression of NCS-
1(E120Q) at the plasma mem-
brane, secretion observed in cells co-transfected with pCMV5-hGH and empty vector was set to 100%, and relative enhancement of secretion with NCS-1 was normalized to control empty vector (normalized enhance-
ment of release). Values shown represent means ± S.E. from five experiments. Statistically significant differences are marked by aster-
isks (*, p < 0.25; **, p < 0.005).

Overexpression of NCS-1 Leads to a Shift in the Dose-re-
sponse Curve Using Phenylarsine Oxide—In chromaffin and PC12 cells, phosphorylation of phosphatidylinositols by PI4Kα is
required for Ca^{2+}-triggered exocytosis (25–27). In these cells, PI4K can be potently inhibited by PAO, resulting in a block of exocytosis (27, 50). Indeed, incubation of the cells with 3 μM PAO for 15 min prior to triggering Ca^{2+}-dependent secretion with ATP resulted in a robust inhibition of hGH release in both vector and NCS-1-transfected cells (Fig. 5A). These PAO con-
centrations are similar to those reported to inhibit granule
secretion and PtdIns(4,5)P_2 production from synaptosomes.

Fig. 3. Localization and amounts of overexpressed NCS-1 and PI4Kα. A, extracts (60 μg) of PC12 cells transfected with various NCS-1 constructs were analyzed by Western blotting. B, overexpression and localization of NCS-1 constructs in NGF-differentiated cells was as-
sessed by immunocytochemistry using anti-NCS-1 chicken polyclonal (22) at a 1:500 dilution to preferentially visualize NCS-1-overexpressing cells. C, extracts (60 μg) of PC12 or COS-7 cells transfected with various PI4Kα constructs were analyzed by Western blotting. D, expression of GFP-tagged N-terminal PI4Kα (PI4K-N-term.) and GFP-tagged, palmitoylated/myristoylated 5’-PtdIns(4,5)P_2-phosphatase (INP51) was visual-
zied in live cells. Scale bar, 10 μm.

Fig. 4. NCS-1 differentially modulates secretion evoked by different secretagogues. Cells were co-transfected in parallel either with pCMV5-hGH and empty control vector (pcDNA3) or with pCMV5-hGH and NCS-1. Representative experiments in which hGH secretion was stimulated for 10 min with control physiological saline (ctrl) and with 300 μM ATP in physiological saline (A), high K^+ (56 mM KCl) solution (B), or with 5 μM ionomycin in physiological saline (C). D, secretion observed in cells co-transfected with pCMV5-hGH and empty vector was set to 100%, and relative enhancement of secretion with NCS-1 was normalized to control empty vector (normalized enhance-
ment of release). Values shown represent means ± S.E. from five experiments. Statistically significant differences are marked by aster-
isks (*, p < 0.25; **, p < 0.005).
FIG. 6. Increase in plasma membrane PtdIns(4,5)P₂ levels upon NCS-1 transfection as imaged with PH-EGFP. Cells were co-transfected in parallel with PH-EGFP and NCS-1, PH-EGFP and NCS-1(G2A), PH(R40L)-EGFP and NCS-1, or PH(R40L)-EGFP and NCS-1(G2A). Two days after transfection, cells were fixed and permeabilized, and EGFP fluorescence was imaged. Fluorescence intensities were quantified using rectangles of identical pixel size centered on 100 EGFP-positive cells for each condition.

A, representative cells expressing NCS-1 and PH-EGFP (left) or NCS-1 and PH(R40L)-EGFP (right). Scale bar, 10 μm.

B, averaged arbitrary fluorescence intensities (means ± S.E., n = 3) from a total of 300 cells each co-expressing empty control vector (pcDNA3) or NCS-1 or NCS-1(G2A) and either PH-EGFP or PH(R40L)-EGFP, respectively. *, p < 0.05.

C, distribution of cells expressing identical plasma membrane-associated (PH-EGFP) or cytosolic (PH(R40L)-EGFP) GFP fluorescence (binned in 0.25 arbitrary units). Open squares and open diamonds, cells co-transfected with NCS-1. Closed circles and closed triangles, cells co-transfected with NCS-1(G2A).

FIG. 5. NCS-1 expression causes a shift in the dose-response curve using phenylarsine oxide. Cells were co-transfected in parallel either with pCMV5-hGH and empty control vector (pcDNA3) or with pCMV5-hGH and NCS-1. A, experiment in which cells were incubated in medium containing either Me₂SO (final 0.5% (v/v)) or 3 mM PAO in Me₂SO (final 0.5% (v/v)) for 15 min before secretion assays were carried out using either control physiological saline (ctrl) or 300 μM ATP in physiological saline. B, experiment in which cells were incubated with 0, 0.3, 1, or 3 mM PAO for 15 min before secretion assays. The amount of secretion in the absence of PAO was set to 100% for both control vector and NCS-1-transfected cells. Results are shown from a single experiment performed in duplicate. Although the amount of inhibition with specific PAO concentrations was variable between experiments, the shift in the dose-response curve upon NCS-1 transfection was reproducible (n = 3 experiments, each performed in duplicate).
Treatment of the cells with PAO did not affect the total levels of hGH (data not shown), suggesting that it specifically blocks Ca\textsuperscript{2+}-dependent secretion evoked by ATP, rather than interfering with hGH expression. Further, PAO blocked exocytosis in a dose-dependent manner in both control and NCS-1-transfected cells. When the levels of hGH release in the absence of PAO were normalized to 100%, inhibition of release was less pronounced in cells expressing NCS-1 as compared with cells expressing control vector (Fig. 5B). These data are consistent with our proposal that NCS-1 expression leads to enhanced levels of active PI4K\textsubscript{II} and thus to a concomitant shift in the PAO dose-response curve of hGH secretion.

Increase in Plasma Membrane PtdIns(4,5)P\textsubscript{2} Levels upon NCS-1 Transfection—As another means to measure enhanced PI4K\textsubscript{II} activity upon NCS-1 expression, we visualized plasma membrane PtdIns(4,5)P\textsubscript{2} levels using a PLC\textsubscript{II} PH domain-GFP (PH-EGFP) construct. The PH domain of PLC\textsubscript{II} specifically binds PtdIns(4,5)P\textsubscript{2} and has been shown to be predominantly associated with the plasma membrane (28, 37, 51). Indeed, PH-EGFP specifically labeled the plasma membrane, and to a lesser extent the cytosol, in live PC12 cells (data not shown). When cells were fixed and permeabilized, cytoplasmic staining was minimal, and PH-EGFP predominantly localized to the plasma membrane (Fig. 6A). The PLC\textsubscript{II} PH domain contains three critical basic residues that interact with PtdIns(4,5)P\textsubscript{2}, including Arg-40 (52). A mutated PH domain construct, PH(R40L)-EGFP (28), did not label the plasma membrane but was entirely cytosolic (Fig. 6A). These two constructs were used in co-transfection experiments with either NCS-1 or the non-functional NCS-1(G2A). The intensity of PH-EGFP staining in cells co-transfected with NCS-1 was counted from 300 individual cells well separated from each other and compared with the intensity of PH-EGFP staining in cells co-transfected with NCS-1(G2A) or cells co-transfected with empty control vector (pcDNA3) (Fig. 6B). NCS-1 expression led to a 25.9.6% increase in PH-EGFP staining as compared with control vector and a 3.7.4% increase as compared with NCS-1(G2A) (Fig. 6B). To exclude that the differences in PH-EGFP staining were because of differences in residual cytosolic PH-EGFP intensities upon NCS-1 transfection, we used the cytosolic PH(R40L)-EGFP as a control. NCS-1 expression did not lead to enhanced PH(R40L)-EGFP staining, suggesting that the observed increase in PH-EGFP staining is specific for PtdIns[4,5]P\textsubscript{2} levels at the plasma membrane imaged by PH-EGFP. Co-expression of PH(R40L)-EGFP with either NCS-1 or NCS-1(G2A) led to a slight decrease in the averaged fluorescence intensities as com-

**FIG. 7.** PAO treatment leads to decrease in plasma membrane PtdIns(4,5)P\textsubscript{2} levels. Cells were transfected with PH-EGFP, and 2 days after transfection, cells were treated with 5 \mu M PAO or Me\textsubscript{2}SO vehicle alone for 15 min prior to fixation. Fluorescence intensities were quantified using rectangles of identical pixel size centered within (for cytosol) or around (for total) 100 EGFP-positive cells for each condition. Distribution of cells expressing identical cytosolic (A) or total (B) PH-EGFP fluorescence (binned in 0.25 arbitrary units) is shown. Open squares, cells treated with Me\textsubscript{2}SO only. Closed circles, cells treated with 5 \mu M PAO in Me\textsubscript{2}SO. C, averaged arbitrary fluorescence intensities (means \pm S.E., n = 3) from a total of 300 cells expressing PH-EGFP in either the absence or presence of PAO, measuring cytosolic (left) and total (right) GFP fluorescence, respectively. *, p < 0.001.

**FIG. 8.** Effect of NCS-1 overexpression on phosphorylation of endogenous lipid substrate in permeabilized PC12 cells. Cells were electroporated with either pcDNA3.1 (C) or NCS-1 (N) constructs. Two days later, cell lysates or membrane fractions were analyzed for [\gamma\textsuperscript{32}P]phosphate incorporation into phospholipids from [\gamma\textsuperscript{32}P]ATP. A, representative TLC analysis of radiolabeled PtdIns(4)P and PtdIns(4,5)P\textsubscript{2} production of entire cell lysates or membrane fractions. B, summary of quantitative data from several experiments (n = 6 for cell lysate, n = 3 for membrane fractions). *, p < 0.04. Phosphorylation of lipids was quantified by phosphorimaging analysis (PhosphorImager; Molecular Dynamics).
pared with control empty vector (pcDNA3) (Fig. 6B), indicative of a competition for the transcriptional/translational machinery of the two co-expressed constructs. This competition was also visible upon co-expression of PH-EGFP and NCS-1(G2A), suggesting that the direct comparison between effects of NCS-1 and NCS-1(G2A) is most appropriate.

Because expression levels in transfection experiments are highly variable, we also plotted PH-EGFP fluorescence intensities binned from the whole population of analyzed cells (Fig. 6C). NCS-1 expression led to a decrease in the number of cells with low amounts of plasma membrane PH-EGFP staining and a concomitant increase in the number of cells with larger amounts of plasma membrane PH-EGFP staining (Fig. 6C).

These data suggest that NCS-1 overexpression leads to a specific increase of PtdIns(4,5)P$_2$ levels at the plasma membrane, in agreement with its proposed role in activating PI4K$\beta$. NCS-1 expression led to a decrease in PH-EGFP staining (Fig. 7A). However, the total amount of PH-EGFP staining in transfected cells treated with PAO and treated for 15 min with PAO prior to fixation was counted from 300 individual cells and compared with the intensity of cytosolic PH-EGFP staining in transfected cells treated for 15 min with Me$_2$SO (Fig. 7B). PAO treatment led to a decrease in the number of cells with small amounts of cytosolic PH-EGFP staining and a concomitant increase in the number of cells with larger amounts of cytosolic PH-EGFP staining (Fig. 7A). However, the total amount of PH-EGFP staining in both cytosol and plasma membrane was not changed upon PAO treatment, indicating that the increase in PH-EGFP staining in the cytosol was accompanied by a decrease in PH-EGFP staining at the plasma membrane (Fig. 7B and C). Overall, treatment of cells with PAO resulted in a 45 ± 6% increase in cytosolic PH-EGFP staining as compared with control Me$_2$SO treatment, indicating that PAO treatment results in a partial knock-off of PH-EGFP staining from the plasma membrane because of a resultant decrease in plasma membrane PtdIns(4,5)P$_2$ levels (Fig. 7C).

Overexpression of NCS-1 Leads to Increased PtdIns(4)P Production—The effect of overexpressing NCS-1 on the phosphorylation of endogenous phosphatidylinositol (61, 62) was also examined in permeabilized PC12 cells. Expression of NCS-1 caused a significant increase in $^{32}$P labeling of PtdIns(4)P and PtdIns(4,5)P$_2$ in cell lysates and membrane fractions, as compared with cells transfected with empty control vector (Fig. 8). The NCS-1-dependent increase in the labeling of PtdIns(4)P was abolished upon incubation with 10 $\mu$M wortmannin (data not shown), indicating that it was because of enhanced type III PI4K$\beta$ activity (Fig. 8). The substantial amount of radiolabeled PtdIns(4)P produced in the absence of NCS-1 overexpression was likely because of the activity of a type II enzyme, which is responsible for a large amount of PI4K activity in membranes of mammalian cells, as it was not sensitive to wortmannin and significantly stimulated with Triton X-100 (data not shown). These data are in agreement with a recent report indicating that endogenous PtdIns(4)P and PtdIns(4,5)P$_2$ levels were increased in a stable NCS-1-expressing cell line as compared with control cells (63), which further validates the present approach in permeabilized cells to measure PI4K$\beta$-dependent changes in PtdIns(4)P and PtdIns(4,5)P$_2$ levels upon transient overexpression of NCS-1.

Novel NCS-1 Mutations Defective in Enhancing Ca$^{2+}$-Dependent Secretion—NCS-1 is structurally similar to recoverin (53, 54) and can functionally replace the activating effect of recoverin on rhodopsin kinase in vitro (55). The residues within the N-terminal region of recoverin (Phe-22, Glu-26, Phe-55, Thr-92) implicated in making contacts with rhodopsin kinase (56) are all conserved in NCS-1. Moreover, the recently solved crystal structure of human NCS-1, which is identical in sequence to the rat homologue, revealed that three of these residues (Phe-22, Phe-55, Thr-92) are solvent-exposed and located in the wide hydrophobic crevice at the surface of NCS-1, which interacts with its protein ligand (54). The fourth, Glu-26, makes important side-chain contacts with helix J, the C-terminal region of NCS-1 that lies adjacent to the hydrophobic crevice and is thought to be important for ligand recognition (54). To determine whether these residues are essential for the function of NCS-1, we generated a series of novel NCS-1 mutations and tested their effects on hGH secretion (Fig. 9). Mutation E26A, which may interfere with the proper positioning of helix J, and mutations F22A and F55A, which may alter the ability of the hydrophobic crevice of NCS-1 to interact with its downstream target, abolished the ability of NCS-1 to enhance regulated secretion (Fig. 9, A and B). Mutation T92A was still functional in enhancing release, suggesting that the effects of F22A and F55A are specific and that not all solvent-exposed...
resides in the hydrophobic crevice interact with a protein ligand. In addition, the localization, overexpression levels, and co-transfection efficiencies of F22A, F55A, and E26A were equivalent to those of wild-type NCS-1 (Fig. 9C) (data not shown), and overexpressed mutant constructs were not degraded (Fig. 9C), indicating that their impaired ability to enhance secretion is not because of mislocalization or lowered expression levels. The in vitro effect of these mutants, together with the structural information mentioned above, suggests that residues Phe-22, Phe-55, and Glu-26 may function to allow PtdIns(4,5)P2 levels and channel activity. Modulation of channel activity may be either direct or indirect through regulating the amount of channels expressed on the cell surface. For example, overexpression of NCS-1 leads to its localization in the cell periphery, in addition to a diffuse distribution throughout the cytoplasm, and to a concomitant increase in Kv4 channel localization at the plasma membrane (18), suggesting that NCS-1 may increase membrane traffic of channel proteins, which by itself may be a PtdIns(4,5)P2-dependent process.

Activation of PI4Kδ by NCS-1 will lead to concomitant de novo synthesis of PtdIns(4,5)P2, which has been shown to be essential for vesicle priming reactions in neuroendocrine cells (25, 26, 29, 58). During priming, PI4K and PtdIns(4,5)P2-5-kinases may act in concert to modify the two newly juxtaposed membranes and contribute to the acquisition of fusion competence. Lipid polymorphism as exemplified by a localized increase in PtdIns(4,5)P2 may have a direct physiological role in the generation of membrane curvature as required during membrane fusion (59). Alternatively, the localized increase in PtdIns(4,5)P2 may allow recruitment of proteins involved in mediating secretion, such as CAPS (31, 60). In summary, our present study indicates that in neuroendocrine cells, the NCS-1-mediated enhancement of secretion is because of enhanced PI4Kδ activity and concomitant increases in plasma membrane PtdIns(4,5)P2 levels.

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