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Irene Domínguez-González, Silvia N Vázquez-Cuesta, Alicia Algaba, F Javier Díez-Guerra. Neurogranin binds to phosphatidic acid and associates to cellular membranes. *Biochemical Journal*, 2007, 404 (1), pp.31-43. 10.1042/BJ20061483 . hal-00478674

HAL Id: hal-00478674

<https://hal.science/hal-00478674>

Submitted on 30 Apr 2010

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NEUROGRANIN BINDS TO PHOSPHATIDIC ACID AND ASSOCIATES TO CELLULAR MEMBRANES

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Synopsis

Neurogranin (Ng) is a 78 amino acid long protein concentrated at dendritic spines of forebrain neurons that is involved in synaptic plasticity through the regulation of calmodulin (CaM) mediated signalling. Ng features a central IQ motif that mediates binding to CaM and is phosphorylated by protein kinase C (PKC). We have analyzed the subcellular distribution of Ng and found that it associates to cellular membranes in rat brain. In vitro binding assays revealed that Ng selectively binds to phosphatidic acid (PA) and that this interaction is prevented by CaM and PKC phosphorylation. Using the peptide Ng(29-47) and a mutant with an internal deletion (Ng-IQless), we have shown that Ng binding to PA and to cellular membranes is mediated by its IQ motif. Ng expressed in NIH-3T3 cells accumulates at peripheral regions of the plasma membrane and localizes at intracellular vesicles that can be clearly visualized following saponin permeabilization. This distribution was affected by phospholipase D (PLD) and phosphatidylinositol 4-phosphate 5-kinase (PIP5K) overexpression. Based on these results, we propose that Ng binding to PA may be involved in Ng accumulation at dendritic spines and that Ng could modulate PA signalling in the postsynaptic environment.

Key words: Neurogranin, phosphatidic acid, calmodulin, protein kinase C, phospholipase D, phosphatidylinositol 4,5 bisphosphate.

Abbreviations used:

Neurogranin (Ng); phosphatidic acid (PA); calmodulin (CaM); protein kinase C (PKC); phospholipase D (PLD); phosphatidylinositol 4-phosphate 5-kinase I α (PIP5KI α); phosphatidylcholine (PC); diacylglycerol (DAG); diacylglycerol kinase (DGK); lyso-phosphatidic acid (LPA); protein phosphatase 1 (PP1); mammalian target of rapamycin (mTOR); phosphatidylinositol 4,5 bisphosphate (PIP2).

INTRODUCTION

Neurogranin (Ng) (also named RC3, p17 or BICK) is a small protein originally identified in rat brain [1-3] that is abundantly expressed in the cerebral cortex, hippocampus, amygdala and basal ganglia. Ng expression develops postnatally in most areas and peaks during the second and third postnatal week in rodents [4], coincident with periods of active dendrite branching, synaptogenesis and the maturation of synaptic plasticity signalling cascades [5]. Ng binds calmodulin (CaM) in the absence or at low levels of Ca^{2+} [6] and is phosphorylated by protein kinase C (PKC) [1]. CaM binding and PKC phosphorylation occur at a central region of the molecule that shows high homology to the IQ motif present in GAP-43, an axonal protein abundantly expressed in developing and regenerating neurons [7]. CaM binding and PKC phosphorylation at the IQ motif are mutually excluding events.

Ng knock-out mice show severe deficits in performing hippocampus-dependent tasks [8, 9]. It has been proposed that these cognitive deficits are caused by the attenuation of Ca^{2+} and Ca^{2+} /CaM-mediated signalling [10] according to the following scheme. In the postsynaptic environment of resting neurons, Ng would be bound to CaM. Following excitatory stimulation, a rise in Ca^{2+} levels and PKC activation would release CaM from Ng stores to participate in Ca^{2+} /CaM-mediated pathways. In this way, the amount of CaM locally available for signalling purposes would be drastically increased after stimulation. Actually, mice lacking Ng have lower amounts of phosphorylated Ca^{2+} /CaM-dependent protein kinase II (CaMKII) in their brains [8, 11], suggesting an attenuated signalling through this pathway. Also, they show a decreased response of protein kinase A (PKA) and PKC to excitatory stimulation [12], which would be most likely related to the marked anomalies found in these mice for LTP-LTD induction [13].

During the last two decades, it has become evident that cellular lipids not only form bilayers to separate compartments, but also participate in signal transduction and events that lead to cellular polarization, endocytosis, exocytosis, etc. Phosphoinositides have received major attention at this respect, but other phospholipids such as phosphatidic acid (PA) are quickly emerging as signalling molecules [14]. The spatiotemporal distribution of cellular PA is tightly regulated by localized synthesis -mediated by phospholipases D (PLDs), diacylglycerol kinases (DGKs) or acyl transferases- and quick and efficient degradation catalyzed by lipid phosphate phosphatases [15]. PA participates in a variety of signalling events. It activates phosphoinositide 4-phosphate 5-kinase (PIP5K) [16] or the mammalian target of Rapamycin (mTOR) [17] and inhibits protein phosphatase 1 (PP1) [18]. It can also act as a binding partner for protein translocation and recruitment, as is the case of Raf-1kinase [19], and has been shown to be important in cytoskeleton remodelling and vesicle trafficking.

Previous immunocytochemical work had established that Ng localizes to neuronal cell bodies and dendrites, distributes in multiple small aggregates (hence its name, Neurogranin) and concentrates at dendritic spines [20-23]. Ng sorting in the somatodendritic compartment can be adequately explained based on the dendritic targeting of its mRNA [24, 25] and regulated local translation [26]. However, its characteristic distribution in small aggregates cannot be solely justified by its interaction with CaM. In the present work, we have analyzed in detail the subcellular localization of Ng and found that substantial amounts of Ng associate to membranes. A search for interacting partners has revealed that Ng selectively binds to PA, and that free CaM or PKC phosphorylation prevents this interaction. Using an optimized immunostaining method, we have shown that Ng concentrates in peripheral and presumptively highly dynamic regions of the plasma membrane and that this localization is altered after experimental manipulation of cellular PA production and distribution. The relevance of the interaction between Ng and PA is discussed in a context of synaptic plasticity.

EXPERIMENTAL

Antibodies, peptides and recombinant proteins

Polyclonal antibodies to Ng were developed in rabbits using the peptide Ng(9-25) covalently linked to Keyhole Limpet Hemocyanin (Imject KLH, Pierce) (Ab205) or recombinant rat Ng (Ab756) as immunogens. Affinity-purified antibodies were obtained from clarified immune sera using affi-gel resins (Bio-Rad) coupled to the relevant immunogens. Polyclonal antibody to GAP-43 (Ab797) was raised in rabbit against recombinant rat GAP-43 and affinity-purified as described above. Monoclonal mouse antibody to CaM was kindly provided by Dr. J. M. McDonald (University of Alabama, Birmingham). Other antibodies were commercially available. The peptides biotin-AAKIQASFRGHMARKKIK and biotin-AAKIQAS(P)FRGHMARKKIK (phosphoserine version), corresponding to the sequence Ng(29-47), were synthesized in an Applied Biosystems peptide synthesizer (model 431A) using fmoc chemistry, purified by gel filtration and HPLC and verified by mass spectrometry. Recombinant rat Ng and GAP-43 were prepared as described [27]. cDNAs of Ng mutants were subcloned into pGEX-2T (Pharmacia) and expressed in Rosetta Competent Cells (Stratagene). Recombinant Ng mutants were purified using glutation-agarose affinity chromatography and thrombin digestion.

Immunocytochemistry

Rats were deeply anesthetized by intraperitoneal administration of a mixture of ketamine, valium and atropine (5:4:1 in volume) and transcardially perfused with phosphate-buffered saline (PBS) for 10 min and then with cold 4% paraformaldehyde (PFA) in PBS for 30 minutes. Brain pieces were post-fixed overnight in the same fixative and 100 μ m sections cut on a vibratome. Endogenous peroxidase activity was blocked with 0.1% H_2O_2 in 0.12M phosphate buffer, pH 7.3 (PB) (20 min) and free-floating sections successively incubated in a) 5% heat-inactivated horse serum (HS), 0.2% (w/v) Saponin in PB for 2 hours, b) affinity-purified anti-Ng antibody (1:500) in the same buffer overnight, c) biotinylated goat anti-rabbit immunoglobulin G (IgG) (1:200) for 2 hours and d) ABC reagent (Vector, Burlingame, CA) for 1 hour. Immunostaining was visualized using 3,3'-diaminobenzidine (DAB) (Sigma) and H_2O_2 in PB. Immunostained sections were either dehydrated and mounted with Eukitt (Merck) for wide field light microscopy or enhanced with osmium tetroxide and cut in an ultramicrotome to obtain semithin sections (1-2 μ m thick) that were observed at the optical microscope and further cut to obtain ultrathin sections (60-100 nm thick) that were analyzed at the electron microscope (EM) (JEOL JEM1010), according to established methods [28]. Images were captured with a Photometrics Coolsnap FX digital camera attached to a Zeiss Axiovert200M microscope or, for EM, with a Gatan BioScan camera.

Ng-CaM binding assay

Serial dilutions of purified recombinant Ng or mutants (250 ng to 10 μ g) were prepared in a volume of 100 μ l containing 12 mM Tris-HCl pH 7.0, 75 mM NaCl, 1 mM EGTA, 2 mM EDTA and 5 mM β -mercaptoethanol and added to 30 μ l of CaM-agarose (Sigma) which had been washed twice in the same buffer. Following incubation during 1 hour at 4°C in thermomixer, non-bound Ng (super) and CaM-bound Ng (pellet) were separated by centrifugation (5.000 xrpm, 1 min). Representative aliquots were analyzed by Western blot and the ratio between bound and non-bound Ng (pellet/super ratio) calculated for each sample.

Protein-lipid overlay assay

All assays were performed at 4°C in the dark. Recombinant Ng or GAP-43 were used to study their binding to phospholipids. To screen for possible interactions, PIP Strips (Echelon Biosciences Inc.) were used according to the manufacturer's protocol. HyBond-C extra nitrocellulose (Amersham) and PA from different sources were used to prepare "in situ" PA-spotted strips. In brief, lyophilized PA was dissolved in methanol:chloroform (1:1) at 1 mM and stored at -80°C (stocks). Working PA solutions were prepared in methanol:chloroform:water (2:1:0.8) and 1 μ l aliquots containing 50, 25 and 12.5 pmol of PA were spotted on membrane strips (Amersham). The spotted strips were dried under nitrogen and left overnight at room temperature. The following day, the strips were blocked with 3% BSA (essentially fatty acid-free) in Tris-

buffered saline (TBS) containing 0.1% (w/v) Tween-20 (TBST) for 60 min at 4°C and incubated overnight at 4°C with gentle rocking in fresh TBST containing 0.5 µg/ml Ng. The day after, the strips were washed 4 times over 40 min in TBST, incubated with Ng antibody (1:20,000) in TBST for 2 hours, washed 10 times during 1 hour in TBST and incubated with HRP-conjugated anti-rabbit antibody (Jackson, 1:12,000) diluted in TBST for 1 hour. This was followed by a final washing step of 12 times in TBST over 60 min and visualization of HRP activity by enhanced chemiluminescence (ECL, Amersham).

Liposomes

Stock solutions of phosphatidylcholine (PC) and PA were prepared in methanol:chloroform (1:1) at 5µg/µl and stored at -80°C. PC and PA from stocks were pipetted into glass tubes containing 100 µl of chloroform to generate mixtures of 125 µg total lipid at different PA:PC proportions (0 to 50% of PA). The glass tubes had been previously washed with ethanol and water and dried in an oven. The PA:PC mixtures were dried under a nitrogen stream and kept one hour more at room temperature. 250 µl of 150 mM NaCl, 10 mM Tris-HCl, pH 7.5 were added to each tube and the liposomes generated by three cycles of 1 min vortex agitation at maximum and two cycles of 1 min in a bath sonicator. At this point, 5 µg of purified recombinant Ng was added to the liposomes and the mixture incubated at 25°C for 30 min with agitation. Incubations were terminated by centrifugation at 200,000x g during 25 min Pellets and supernatants were separated and their Ng content analyzed by Western blot.

Expression Vectors

The cDNAs for Ng, Ng-C3,4,9S, Ng-S36A and Ng-S36D were a generous gift of Dr. Dan Gerendasy (Scripps Institute, La Jolla). The cDNA for Ng-I33Q was donated by Dr. Dan Storm (University of Washington). Ng cDNAs were subcloned into pcDNA3 (Invitrogen) for protein expression. Ng-IQless and double-Ng mutant cDNAs were made by PCR cloning in pcDNA3. Ng-IQless has an internal deletion between residues 30 and 45 of the rat sequence. PH-PLCd1-EGFP was a kind gift of Dr. Tamas Balla (NIH, Bethesda). PIP5KIα-myc-pCMV5 was a kind gift of Dr. Helen Yin (University of Texas). Constructs for phospholipase D (PLD) expression p-EGFP-C1-mPLD2 and p-EGFP-C1-hPLD1b were kindly provided by Dr. Michael A. Frohman (Stony Brook).

Cell culture, transfection and immunofluorescence

NIH-3T3 cells were grown in DMEM supplemented with 10% fetal calf serum (FCS), 1 mM glutamine and antibiotics (penicillin and streptomycin at 50 U/ml). For transfection, 1.5×10^5 cells were plated in P35 dishes containing (or not) 4 round coverslips and the following day transfected with Lipofectamine 2000 (2:1 ratio) in OptiMem for two hours and a half. Then, the cells were returned to their normal growth

medium and processed 24 hours later. Coverslips used for immunofluorescence (IF) were cleaned with 65% nitric acid overnight, extensively washed with distilled water and heat-sterilized. Affinity-purified antibodies to Ng were used at 1:500. Two different methods were used for IF. *Conventional IF method:* The whole procedure was performed at room temperature. After a quick rinse with PBS, cells were fixed with 4% PFA in PBS for 20 min, washed three times with PBS and incubated with 0.1 M glycine pH 8.0 to inactivate free aldehydes. Blocking non-specific binding and permeabilization was achieved by incubating in blocking buffer (5% heat-inactivated horse serum, 0.1% (w/v) Nonidet P-40 (NP-40) in PBS) for 30 min. Primary and secondary antibody incubations were done in blocking buffer for 60 and 45 min, respectively, and followed by a three times 5 min washing step in PBS. Finally, coverslips were sequentially rinsed in distilled water and ethanol and mounted in Mowiol after the ethanol had completely evaporated. *Cool IF method:* Cells grown on coverslips in multiwell (x24) plates were quickly washed with Hank's medium (137 mM NaCl, 5.3 mM KCl, 0.45 mM KH₂PO₄, 0.35 mM Na₂HPO₄, 1.25 mM CaCl₂, 0.8 mM MgSO₄, 1 mM NaHCO₃, 1 mM pyruvate, 0.6% D-glucose and 10 mM HEPES pH 7.3) and fixed by addition of 1 ml of 4% PFA in Hank's at room temperature. After 10 min of fixation, 1 ml of ice-cold fixative was added slowly to each well and fixation continued at 4°C for an additional 30 min. All subsequent steps were performed at 4°C. Cells were quickly rinsed with 0.2% (w/v) Saponin in PBS (PBS-S), incubated with 50 mM glycine in PBS-S for 10 min and rinsed again with PBS-S. Incubation with blocking buffer (1% heat-inactivated horse serum, 0.1% BSA in PBS-S) was extended for 30 min. Primary (90 min) and secondary antibody (45 min) incubations in blocking buffer were followed by three 5 min washing steps in PBS. In many occasions cells were counterstained with Phalloidin-Coumarin or Phalloidin-Tetramethylrodamine (1:1,000) for 10 min in PBS. Finally, coverslips were rinsed with distilled water; drained and let them dry before mounting in Mowiol. Visual inspection and image collection was done using 63x PlApo 1.4 NA and 100x PlApo 1.4 NA objectives in a Zeiss Axiovert200M microscope equipped with a Photometrics Coolsnap FX monochrome camera and MetaMorph 6.1 software (Universal Imaging). Digital image processing (averaging and background subtraction) and montage were done using ImageJ software (Rasband, W.S., National Institutes of Health, Bethesda, MA, USA, <http://rsb.info.nih.gov/ij/>, 1997-2006).

Extracts, density fractionation and western blots

Rats were sacrificed according to the EEC rules and their brains quickly removed and cleaned from meninges and blood clots. All subsequent steps were performed at 4°C. For density fractionation, rat forebrains (without cerebellum and brain stem) were homogenized (1:10, w/v) in 0.32 M sucrose, 1 mM EDTA and 10 mM MOPS pH 7.4 and centrifuged at 1,300 xg (10 min) to obtain a pellet (P1) and a supernatant (S1) that was again centrifuged (15,000 xg, 15 min). The supernatant (S2) was centrifuged at

150,000 xg (60 min) to obtain a soluble (S3) and particulate (P3) fraction. The pellet (P2) was resuspended in homogenization buffer, layered on top of a discontinuous sucrose gradient consisting of 4 ml of 1.2 M sucrose and 3.5 ml of 0.8 M sucrose in the same buffer. After centrifugation (100,000 xg, 30 min, swinging bucket), the material accumulated at the upper (Mye) and lower (Syn) interphases was collected. For hypotonic extraction, tissue was homogenized in a buffer containing 10 mM Tris-HCl pH 7.4, 2 mM EDTA, 10 mM 2-mercaptoethanol, 1 mM PMSF, 1 µg/ml Aprotinin, 1 µg/ml Leupeptin and 1 µg/ml Pepstatin. Homogenates were then centrifuged at 1,200x g (10 min) to obtain a pellet (P1) and a supernatant (S1). The pellet P1 was quickly resuspended in homogenization buffer plus 0.1% (w/v) Triton X-100 (TX-100) and centrifuged again to obtain a soluble (N sup) and a particulate fraction (N pel). The supernatant S1 was centrifuged at 100,000x g (40 min) to obtain a particulate (P3) fraction and a soluble (S3) fraction. Detergent-insoluble glycosphingolipid and cholesterol-rich complexes (DIGs) were prepared from rat brain homogenized in ice-cold lysis buffer (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA and 1% Triton X-100) as described [29]. The homogenate was brought to 40% sucrose (w/v) in a final volume of 4 ml and placed at the bottom of a discontinuous gradient of sucrose composed of two more layers: an intermediate 6 ml layer (30% sucrose) and an upper 2 ml layer (5% sucrose). After equilibrium centrifugation at 100,000 xg, the interface between 5 and 30% sucrose was collected by aspiration and separated as the DIG fraction. The fraction at the bottom (40% sucrose) was taken and defined as the Triton X-100 soluble fraction. For cell extracts, cells were scraped and homogenized (250 µl/P35) in cold medium (10 mM Tris-HCl, pH 7.5, 2 mM EDTA, 10 mM 2-mercaptoethanol, 1 µg/ml pepstatin, 1 µg/ml aprotinin, 1 µg/ml leupeptin and 1 mM PMSF) and centrifuged at 1,000x g for 10 minutes (4°C). The pellet (P1) was separated and the supernatant centrifuged again at 100,000x g for 40 min (4°C) to obtain a soluble fraction (S3) and a particulate fraction (P3). Final resuspension volumes were identical for all fractions. Aliquots from different fractions were mixed with electrophoresis sample buffer, heated at 90°C for 1 min, separated by SDS-PAGE and processed for Western blot as described [27]. Polyclonal antibodies to Ng were used at 1:12,000. Immunoreactive bands were visualized using HRP-conjugated secondary antibodies and enhanced chemiluminescence detection (ECL kit, Amersham).

General Methods

Restriction-enzyme digestions, DNA ligations, site-directed mutagenesis and other recombinant DNA procedures were performed using standard protocols. All DNA constructs were verified by DNA sequencing.

RESULTS

Neurogranin associates to intracellular membranes in neurones. Previous studies have shown that Ng is typically distributed in small aggregates throughout the somatodendritic compartment of neurons [20, 21, 23]. In the present study, we confirmed these data on immunostained sections, using light (Figure 1A) and electron microscopy (EM) (Figure 1B) and additionally found significant amounts of Ng localizing at intracellular membranes (Figure 1B). This observation prompted us to study the association of Ng with cellular membranes. Thus, rat brains homogenized in isotonic sucrose were density fractionated and their Ng content analyzed by Western blot. More than 50% of brain Ng was recovered in the high-speed supernatant (S3) (Figure 1C). However, Ng is also present in the synaptosomal (Syn) fraction and, more abundantly, in the nuclei-enriched fraction (P1). Since, as seen at the light and electron microscope, Ng is not so prevailing within nuclei, and since other non-nuclear proteins, such as GAP-43, tubulin and synaptophysin, are also abundantly recovered at P1 fractions, we analyzed Ng presence at P1 fractions with more detail. Rat brain homogenates were prepared in hypotonic saline and the low-speed pellets (P1) resuspended in the same buffer with 0.1% (w/v) TX-100 and quickly centrifuged again to obtain, a nuclear pellet (N pel) and a nuclear soluble fraction (N sup). As shown in Figure 1D, Ng in the P1 fraction is almost totally extracted after resuspension with TX-100. Similar results were obtained for GAP-43, tubulin and synaptophysin (not shown), indicating that these proteins are not really localized within nuclei but have been retained in associated membranes. Figure 1C also shows that more than 20% of brain Ng is recovered in the synaptosomal fraction. When synaptosomes were broken by hypotonic shock, Ng remained tightly associated to membranes. Ng could only be released from these membranes with detergents (TX-100, 0.1% w/v) or chaotropic agents (6M Urea) (Figure 1F), but not by changes in ionic strength or pH (not shown). These results indicate a strong interaction between Ng and synaptosomal membranes that could be mediated by lipids, membrane proteins or both. Finally, to find out if Ng was part of lipid rafts enriched in sphingolipids and cholesterol, detergent-insoluble glycosphingolipid and cholesterol-rich complexes (DIGs) were isolated from rat brains. As shown in Figure 1E, Ng is not present in DIGs, unlike GAP-43.

Ng binds to phosphatidic acid. To date, the only Ng-interacting protein known is CaM [6, 30-32]. However, Ng characteristic distribution within neurons and tight attachment to synaptosomal membranes suggest additional interactions. Using Ng immunoprecipitation and cell-permeable cross-linkers, we were unable to identify additional binding partners. We then explored the possibility of a direct interaction between Ng and membrane lipids using a lipid-protein overlay assay (PIP strips, Echelon). Figure 2A shows that Ng selectively binds to PA whereas GAP-43, which shares with Ng a similar IQ motif, does not

bind to PA but to several other phosphoinositides. To discard the possibility of non-specific binding, two different affinity-purified Ng antibodies were used and identical results were obtained. To determine the specificity and affinity of the interaction, we prepared our own PA-spotted Hybond-C nitrocellulose strips using dipalmitoyl-PA, PA from egg yolk and dioleoyl-PA. We found that Ng binds to all of them, but shows lower affinity for dioleoyl-PA (not shown). This result may be related to the different adsorption of the lipids to the nitrocellulose support. From assays using several concentrations of Ng and egg yolk PA, we calculated the affinity of the interaction ($K_D = 83 \pm 16$ nM, mean \pm SEM, $n=4$) (Figure 2B). Since Ng concentration within neurons is estimated at 10 μ M [10] it could be stated that, in the absence of competing interactions, most Ng would be complexed to PA. Next, to investigate the forces involved in stabilizing the interaction we tested the effect of ionic strength and pH. Figures 2C and 2D show that Ng binding to PA is maximal at physiological salt concentrations and peaks at pH values between 8,0 and 8,5. These results indicate that, electrostatic forces presumptively established between the phosphate group of PA and basic sequences of Ng are important to stabilize the interaction, although the concurrence of hydrophobic interactions cannot be ruled out.

The IQ motif, a central stretch 15-20 amino acid long, concentrates most of the basic residues of Ng. This motif is also the binding site for CaM and hosts the PKC phosphorylation site (Ser36) [1]. Therefore, we wished to know if CaM or phosphorylation by PKC interfered with the interaction. Figure 3A shows that Ng preincubation with increasing concentrations of CaM substantially reduced its binding to PA, indicating that Ng-CaM and Ng-PA interactions are mutually exclusive. To check the phosphorylation effect, purified recombinant Ng was phosphorylated with PKC and the reaction mixture passed through a CaM-Agarose column to remove non-phosphorylated Ng. Since only non-phosphorylated Ng binds CaM, the flow-through contains exclusively phosphorylated Ng. Figure 3B shows that phosphorylated Ng displays less affinity for PA than non-phosphorylated Ng, suggesting that phosphorylation interferes with the interaction. The Ng antibodies used were insensitive to the phosphorylation state of Ng. To evaluate the contribution of the IQ motif, a peptide corresponding to the sequence Ng(29-47) was synthesized and biotinylated at the N-terminus. As shown in Figure 3C, the biotin-IQ peptide binds to PA in a saturable fashion whereas the same peptide phosphorylated at Ser36 (biotin-IQ-P) did not display significant binding. The relative affinity of the peptide for PA is lower than Ng's, suggesting that either the spatial conformation and/or additional Ng residues could play a role in the interaction. The possibility of cooperative binding between Ng oligomers and multiple PA molecules should not be disregarded either. To confirm the data obtained with the lipid-protein overlays we used liposomes, which more faithfully reconstitute the physiological environment of protein-lipid interactions. Using liposomes made of phosphatidylcholine (PC) alone or mixtures of PA and PC, we found that Ng only binds to liposomes that

contained PA (Figure 3D, upper panel). Maximal levels of Ng binding were obtained with liposomes containing dioleoyl-PA or egg yolk PA, whereas less binding was observed using liposomes containing dipalmitoyl-PA (not shown). This could be explained by different surface exposure of the PAs in the liposomes. We then checked if CaM could compete with PA for Ng binding or if phosphorylated Ng would bind to liposomes containing PA. As shown in Figure 3D (lower panel), pre-incubation with CaM prevented Ng binding and phosphorylated Ng did not bind to PA-containing liposomes, in good agreement with data obtained previously. In summary, using different preparations, we have shown that Ng selectively binds to PA and that the interaction is prevented by CaM and inhibited by PKC phosphorylation.

Ng associates to intracellular membranes in NIH-3T3 cells. To study the physiological relevance of Ng association to cellular membranes we looked for cell lines with Ng expression, but did not find any. Primary cultures of hippocampal or cortical neurons show very low Ng expression, even after 2 weeks in vitro [33]. Therefore, we transfected a variety of commonly used cell lines, analyzed their Ng expression and decided to use NIH-3T3 cells since expression was not very high and subcellular distribution resembled that observed in tissue (Figure 4A). Ng in NIH-3T3 cells shows a typical intracellular punctate labelling and is observed in small intracellular vesicles and, more seldom, at peripheral dorsal ruffles and edges of lamellipodia. The peripheral localization was not consistently reproduced between experiments. Since Ng is more a peptide than a protein (78 amino acid long), we thought that some Ng could be lost during the fixation and/or permeabilization steps. To check that, we compared different IF protocols and found that cooling the cells during fixation and maintaining the procedure thereafter at low temperatures (4-10°C) is fundamental to fully reveal Ng peripheral localization (see Experimental Procedures). Using this method, Ng antibodies strongly labelled plasma membrane ring-shaped structures (PMRSSs) located at the cell periphery (Figure 4B, Supp. Figure 1). These ring-shaped concave structures resembled open craters at the dorsal plasma membrane that formed at the cellular edges (Figure 4E). Ng-labelled PMRSS were more abundant in cells with moderate to high Ng expression, particularly present in motile elongated cells, and less abundant in contact-inhibited cells. All Ng expressing cells still exhibited the typical intracellular punctate labelling, although sometimes the intense labelling of PMRSSs made it difficult to notice. Additionally, it was common to observe Ng labelling lamellipodia edges, short membrane ruffles and tips of trailing tails of motile cells. This labelling pattern is specific since it was not seen in non-transfected cells and could be reproduced with any of two different Ng antibodies. To investigate the association of Ng to cellular compartments, we analyzed its distribution after “in situ” permeabilization with saponin. Saponin (0.2% w/v), added to live cells, slowly and non-uniformly extracts cellular membranes due to its avidity for cholesterol. Thus, during saponin permeabilization, strictly soluble and

cytosolic proteins quickly diffuse out of cells, whereas membrane or cytoskeleton-associated proteins are retained or released more slowly. For these experiments, GFP was always co-expressed with Ng and used as internal reporter of a typically soluble protein. Fifteen seconds after saponin addition, GFP was completely washed out from transfected cells (not shown), whereas Ng labelling was still strong and displayed a granular pattern (Figure 4C). After 60 seconds of permeabilization, a portion of Ng still remained attached and visible, associated to multiple disseminated spots throughout the cytosol (Figure 4F). Longer extractions (> 2 min) resulted in total loss of Ng. When non-ionic detergents such as Nonidet P-40 (NP-40) or TX-100 (also at 0.2% w/v) were used instead of saponin, both GFP and Ng were totally extracted after 15 secs of permeabilization (not shown). These results again point to an association between Ng and cellular membranes. To support this, homogenates of transfected cells were density fractionated and the content of Ng and GFP analyzed by western blot. As expected, minor but significant amounts of Ng were found in the particulate fractions P1 and P3, whereas GFP was only found in the soluble fraction S3 (Figure 4D).

The IQ motif is involved in Ng translocation to the plasma membrane. We next wished to find out the region of the Ng molecule responsible of its peripheral localization. Initially, we used several Ng mutants with mutations at Ser36 and N-terminal cysteines, and found that Ng-S36A, Ng-S36D and Ng-C3,4,9S localize at the plasma membrane in the same way as Ng does. Ng-S36A and Ng-S36D mutants are meant to mimic the non-phosphorylated and phosphorylated forms of Ng, respectively. Therefore, we reasoned that if Ng binding to PA is responsible of association to membranes, then Ng-S36D should be cytosolic. Another explanation could be that Ng mutants bind PA with equal affinity that Ng. To test that, recombinant Ng-S36A, Ng-S36D and Ng-C3,4,9S were purified and their binding to PA compared to that of Ng in protein-lipid overlays. No affinity differences were found between Ng and the mutants, thus suggesting that Ser36 and Cys3,4,9 residues are not directly involved in Ng-PA interaction. Mutagenesis at phosphorylatable residues is frequently used to study the physiological relevance of particular phosphorylation sites. However, it is not uncommon to find that mutations of Ser/Thr to Asp do not reproduce the behaviour of the phosphorylated protein. In fact, it has been shown that although phosphorylated Ng shows negligible binding to CaM, Ng-S36D displays significant binding to CaM, whereas Ng-I33Q, another unrelated mutant, is unable to bind CaM [32]. We confirmed these data by comparing the affinities of the Ng mutants for CaM “in vitro”. Figure 5 (lower panel) shows that Ng-S36D still retains a substantial amount of binding to CaM, whereas Ng-I33Q does not bind to CaM at all. Then, to confirm whether Ng IQ motif is involved in Ng peripheral localization, we made two additional mutants: one with a deletion of 15 aa corresponding to the IQ motif (Ng-IQless) and another with two Ng molecules linked in tandem (double-Ng). Figure 5 (upper panel) shows that both Ng and Ng-I33Q have a

similar distribution characterized by its peripheral localization and PMRSSs labelling. Ng-IQless, on the other hand, displayed a typical punctate intracellular distribution with no peripheral labelling. The double-Ng mutant showed a characteristic distribution featuring an abundant localization throughout the plasma membrane. “In situ” permeabilization with saponin during 60 sec. completely washed out Ng-IQless from transfected cells, whereas Ng-I33Q and double-Ng displayed the typical spotty distribution found earlier for Ng (not shown). Based on these results, we propose that the IQ motif is involved in Ng translocation to the plasma membrane.

Phospholipase D and PIP5K α overexpression alters Ng intracellular distribution. Having identified and characterized the interaction between Ng and PA, we were interested to investigate its physiological relevance. PLD1 and PLD2 catalyze PA production from PC in mammals and, along with DGKs constitute the major source of cellular PA for signalling purposes [15]. To analyze the effect of an increased production of cellular PA, we overexpressed GFP-tagged PLDs and analyzed Ng distribution. PLD2 and, to a lesser extent, PLD1 overexpression visibly altered Ng distribution (Figure 6). Following PLD overexpression, Ng turned from labelling multiple and small intracellular puncta to a distribution characterized by bigger aggregates disseminated within the cytoplasm or a distribution where practically all Ng was concentrated in one strongly labelled peripheral patch. The patches resembled previously observed PMRSSs, but now appeared to be practically filled with Ng. After “in situ” permeabilization, Ng was observed in intracellular vesicles that were bigger and less numerous than those found in cells expressing only Ng (see Figure 4). PLD2 and PLD1 localized to either the plasma membrane or intracellular vesicles, respectively, even after “in situ” permeabilization, according to what has been already described [34, 35]. No colocalization could be detected between Ng and PLD1 or PLD2. It has been shown that PA and PIP2 production are mutually and positively regulated, for example in membrane ruffles, through cross-activation of PIP5K α and PLD with PA and phosphatidylinositol 4,5 bisphosphate (PIP2), respectively [36]. It is also known that PLD2 has a high catalytic activity which is almost exclusively dependent on PIP2 [37]. To find out how an increase in cellular PIP2 affects Ng distribution we overexpressed PIP5K α . As illustrated in Figure 7 (upper panel), no Ng-labelled PMRSSs were observed in PIP5K α expressing cells. Instead, Ng labelled multiple small protrusions or blebs decorating the dorsal surface of the plasma membrane where PIP5K α was also observed. When Ng was coexpressed along with PIP5K α and PLD2, the same peripheral distribution was found all three proteins (Figure 7, lower panel). Since the interaction between PIP5K α and PLD2 has been clearly documented [38], it is suggested that Ng localization in PIP5K α overexpressing cells is mediated by local generation of PIP2 and upregulation of PLD2 activity.

DISCUSSION

In recent years, the study of Ng participation in neuronal plasticity events has been addressed from multiple approaches [12, 39-44]. In most of them, the general understanding that Ng solely acts as a local CaM chelator concentrating and releasing CaM in a calcium and PKC activity dependent fashion has dominated the discussions. Intracellular signalling not only depends on protein modification but also on restricted subcellular localization and regulated translocation. This is especially relevant in highly complex and morphologically asymmetrical cells such as neurons, where compartmentalization is fundamental for proper function. In the present study, we have undertaken a different approach that relies on the analysis of Ng subcellular localization in a variety of conditions and environments.

Using immunocytochemistry combined with EM, we have confirmed that Ng typically distributes in small aggregates and strongly labels intracellular membranes in neurons. Ng presence in aggregates has been previously observed by other authors [1] and could be related to its strong tendency to form disulfide-linked oligomers. Ng could also be part of multimeric complexes of yet undefined composition and functionality. Regarding membrane association, earlier work had already reported Ng at membranes [20-22, 30] although no binding partners were identified. Here, we show that Ng tightly associates to synaptosomal membranes and selectively binds to PA “in vitro”. Since no other binding partners were found, we propose that Ng binding to PA is required for membrane association. In protein-lipid overlays, Ng binding to PA is stabilized by ionic interactions. However, Ng association to synaptosomal membranes is not equally affected by changes of ionic strength or pH. Therefore, we believe that additional interactions should be involved. Since non-ionic detergents effectively extracted Ng from synaptosomal membranes, we propose that these interactions could be of hydrophobic nature. Further, cellular Ng could interact with membranes as an oligomer and cooperatively bind to PA clusters or be further assembled in a multimeric complex at the PSD. Our data also indicates that the IQ motif mediates Ng binding to PA. There are several proteins whose PA-binding motifs have been identified [45, 46]. Although there is no strict PA-binding consensus sequence, most commonly, PA-binding motifs are short sequences that contain one or more basic residues. Ng IQ motif is a short (15-20 amino acids) amphiphilic (basic and hydrophobic) central region of the molecule that makes a good candidate for a PA-binding motif. Since Ng IQ motif is also the site of CaM binding and PKC phosphorylation, this finding has interesting regulatory implications.

Evidence supporting Ng association with membranes was also obtained in NIH-3T3 cells. In these experiments, the design of an optimized IF method was determinant to see Ng accumulations at the cell

periphery (PMRSSs). The morphology and localization of Ng-labelled PMRSSs lead us to think that they could be related to macropinocytic vesicles originated in ruffling areas that collapsed upon cell fixation [47]. Further, “in situ” permeabilization experiments demonstrated that Ng, although mostly soluble, also associates to multiple intracellular vesicles. Looking for the domains involved in membrane association, we found that Ng-IQless, a mutant without IQ motif, does not localize at the plasma membrane and completely disappears after “in situ” permeabilization. Further, double-Ng, a mutant that features two Ng molecules linked in tandem and consequently two IQ motifs, displays a plasma membrane localization even more evident than Ng. These observations suggest that the IQ motif is important in defining Ng intracellular distribution. On the other hand, Ng-I33Q, a mutant unable to bind CaM, is also widely localized at the plasma membrane with a distribution similar to Ng. Since the IQ motif is the site of CaM interaction, it can be concluded that CaM is not necessary for Ng association to membranes. This is in good agreement with our previous “in vitro” results showing that Ng binding to CaM and to PA are mutually exclusive. Evidence supporting “in vivo” interaction between Ng and PA came from co-expression experiments. Expression of PLD2-GFP and, to certain extent, PLD1-GFP induced visible Ng accumulation at the plasma membrane and localization at bigger intracellular vesicles observed after “in situ” permeabilization. Local PA and PIP2 levels at the plasma membrane are closely linked through cross-activation of PIP5KI α and PLD2. In cells overexpressing PIP5KI α , Ng localizes at the plasma membrane labelling multiple miniprotusions, many of them containing also PIP5KI α . When PLD2 is overexpressed in these cells, a high degree of colocalization is observed among PIP5KI α , PLD2 and Ng. This suggests that PIP5KI α recruits PLD2 to the miniprotusions and stimulates its activity by local production of free PIP2. As a result, Ng would be trapped in these sites by PA locally synthesized by PLD2. In summary, based in our results, we propose that Ng dynamically translocates between intracellular compartments and the plasma membrane and that newly synthesized PA, most likely derived from PIP2-stimulated PLD, is an essential factor in regulating its subcellular distribution.

Assessing the physiological relevance of the interaction between Ng and PA is not a straightforward task, mainly because very little is known of the relevance of PA as a second messenger in a neuronal context [14]. Most likely, intraneuronal distribution of PA would also be tightly regulated by multiple synthesizing and degrading enzymes and, possibly, PA-binding proteins. Fusions of fluorescent proteins with known PA-binding domains (Raf1c, PP1c γ) have failed to report “in vivo” PA distribution (personal comm. of Dr M. Frohman, Stony Brook). With the available data, we could speculate on the functional relevance of Ng-PA interaction at least in two directions. First, PA could act as a membrane anchor to locally recruit and concentrate Ng at specific sites in dendrites. In this way, Ng would benefit from the dynamic and tightly regulated spatiotemporal distribution of PA. This could also explain the selective concentration of Ng at

dendritic spines and post-synaptic densities (PSDs), in spite of being a predominantly cytosolic protein [21, 22]. Second, since newly-synthesized PA has a short half-life, binding to Ng could prevent its degradation and contribute to create a PA reservoir that could be quickly, locally and massively released, for example, after Ng phosphorylation by PKC. Ng, due to its small size and high intracellular mobility, would be a suitable molecule to realize this task. In this scenario, Ng would be an important element to potentiate PA-dependent signalling.

Ng knockout mice suffer from learning and memory deficits. Our current knowledge on learning and memory at the molecular level is based on what we know about LTP and LTD mechanisms, which dictate the signalling context that leads to neurotransmission amplification or attenuation. The balance between kinase and phosphatase activity is crucial for the regulation of the LTP/LTD switch, and CaMKII and PP1 are two key actors in this interplay. Much attention has been paid to Ng ability to release free CaM and its effect on Ca-CaMKII activity [44, 48, 49]. However, not only kinases are important for LTP. Inhibition of phosphatase activity has been demonstrated at least as powerful as kinase stimulation to promote LTP [50, 51]. In this context, it is suggestive to find out that PP1 γ , the predominant PP1 isoform in the postsynaptic compartment [52], is potently inhibited by PA [18]. Therefore, Ng participation in synaptic plasticity mechanisms could be as illustrated in Figure 8. Under resting conditions, most Ng would be associated to CaM (cytosol) or PA (membranes). Following excitatory stimulation, intracellular Ca²⁺ rise and activation of PKC would release CaM and PA from Ng at different sites. CaM would activate CaMKII and promote its autophosphorylation, whereas PA would inhibit PP1 γ activity and prolong the effects of LTP. In the absence of Ng, no CaM or PA would be sufficiently concentrated at the postsynaptic site and a limited CaMKII activation and PP1 γ inhibition would lead to a diminished potentiation. From the evidence accumulated using knockout mice, it seems clear that Ng deficit has a big impact on cognition, spatial learning and memory tasks. This should be recognized as a good opportunity to study the cellular and molecular basis of learning and memory in an animal model without undesired interferences. A more precise knowledge of Ng localization, specific interactions and signalling cascades involved will contribute to a better understanding of the mechanisms of neuronal plasticity and the molecular basis of learning and memory processes.

ACKNOWLEDGEMENTS

This work was supported by a grant from the Spanish Ministry of Science and Technology (BFI2002-01581). We thank “Fundación Ramón Areces” for institutional support. Irene Domínguez-González was recipient of a fellowship from the Comunidad Autónoma de Madrid (CAM).

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FIGURE LEGENDS

Figure 1.- Ng is associated to neuronal membranes in rat brain

Brains from young adult Wistar rats were processed for immunocytochemistry as described in Methods. (A) (left): stereomicroscope print showing half a coronal section displaying abundant Ng staining at the hippocampus, cerebral cortex and amygdala and none in the thalamus. (right) bright field print showing Ng immunostaining distribution across somatosensory cortical layers. (B): Electron microscopy prints showing typical Ng immunostaining of cortical pyramidal neurons. Ng is distributed as small aggregates along an apical dendrite and frequently labels the cytoplasmic face of intracellular membranes. (C): Brains were density fractionated in isotonic sucrose as described in Methods. Aliquots representing equivalent portions of the fractionated compartments were processed by Western blot using specific antibodies. (D): Brains were density fractionated in hypotonic saline as described in Methods. Aliquots representing equivalent portions of the fractionated compartments were processed by Western blot. Most of Ng present at the P1 fraction is recovered soluble after TX-100 extraction. (E): Triton X-100 insoluble (lipid rafts) and soluble fractions were obtained as described in Methods and analyzed by Western blot. No Ng could be detected in the lipid raft fraction, whereas GAP-43 was clearly partitioned between TX-100 soluble and insoluble fractions. (F): Synaptosomes from sucrose density fractionation were washed with isotonic sucrose and homogenized in 20 ml of a solution containing 1 mM EDTA, 10 mM MOPS, pH 7.4. After stirring for 10 min at 4°C, the mixture was centrifuged (15.000 xg, 10 min, 4°C) and the pellet resuspended and frozen in the same buffer at 1.5 mg/ml total protein. For assays, aliquots of synaptosomal membranes were thawed, centrifuged, resuspended in the same buffer with different additions, incubated for 30 min at 4°C with gentle stirring and centrifuged again. Aliquots representing equivalent portions of supernatant (S) and pellet (P) were analyzed by Western blot.

Figure 2.- Ng binds to PA “in vitro”

(A) PIP-strips (Echelon Biosciences Inc) were blocked with 3% fatty acid-free BSA and incubated overnight with recombinant Ng or GAP-43 at 0.5 µg/ml. After extensive washing with TBS/0.1% Tween-20, anti-GAP-43 (797) or anti-Ng (756) affinity-purified antibodies were used to analyze protein content. The experiments were independently performed at least three times obtaining identical results. A representative blot is shown. (B): HyBond-C nitrocellulose strips were spotted with different amounts of egg yolk PA as described in Methods and incubated with different concentrations of Ng. Ng-PA binding was quantified by densitometry of films exposed during different times and expressed in arbitrary units. (C): The effect of ionic strength on Ng binding to PA was analyzed by changing NaCl concentration during overnight incubations with Ng as indicated. (D): The effect of pH on Ng binding to PA was

analyzed using a Tris-Glycine buffer during overnight incubations with Ng. Results are means \pm SEM of at least three independent experiments.

Figure 3.- Regulation of Ng binding to PA

(A): Ng binding to PA was analyzed in protein-lipid overlays as described, except for Ng was previously incubated with different amounts of CaM (30 min, 25°C) before the mixture was added to egg yolk PA spotted strips. (B): Recombinant Ng was phosphorylated by PKC and separated from non-phosphorylated Ng by affinity chromatography in CaM-agarose columns. Binding of Ng (●) and phospho-Ng (■) was assayed as described. (C): Binding of biotin-IQ peptide (●), corresponding to the sequence Ng(29-47), and its phosphorylated form (biotin-IQ-P) (■) to egg yolk PA spotted strips was analyzed using the method described for protein-lipid overlay assays, except for the primary and secondary antibodies were substituted by an incubation with HRP-labelled Streptavidin (1:2.000) for 1 hour. Results in Figures A, B and C are means \pm SEM of at least three independent experiments. (D) Liposomes of the indicated composition were prepared and used immediately as described in Methods. To analyze CaM competition, 5 μ g of CaM were added along with Ng to the binding mixture. Typical Western blots representative of three independent experiments were shown.

Figure 4.- Ng subcellular distribution in NIH-3T3 cells

NIH-3T3 cells were transfected with Ng alone or in conjunction with GFP. (A): Typical distribution of Ng in cells processed with the conventional IF method, showing a characteristic punctate intracellular labelling. (B, E): Ng distribution in cells processed with the cool IF method. Ng accumulates at ring-shaped structures that appear to form at the cell periphery. (C, F): Ng-transfected cells were “in situ” permeabilized with 0.2% saponin for 15 sec (C) or 60 sec (F) and then processed with the cool IF method. Note the clustered distribution of Ng-labelled granules that spread out from the cell after 15 sec of permeabilization and the spotty vesicular distribution observed after 60 sec. Bars represent 25 μ m. (D): Cellular extracts from Ng and GFP transfected cells were density fractionated and analyzed by Western blot. Note that while GFP is completely recovered in the S3 fraction, visible amounts of Ng are present at P1 and P3 fractions.

Figure 5.- Ng localization at the plasma membrane is mediated by the IQ motif.

(Upper panel) NIH-3T3 cells were transfected with several Ng mutants as indicated in the panels. Cells transfected with Ng and Ng-I33Q displayed a typical distribution with strongly labelled craters, whereas Ng-IQless labelling was limited to intracellular puncta. Double-Ng distribution was characterized by its abundant presence at the plasma membrane. Bars represent 25 μ m. (Lower panel) Binding between CaM-

agarose and several Ng mutants was assayed as described. The results are expressed as the ratio between bound (P) and unbound (S) Ng in each assay. Note that Ng, Ng-S36A and Ng-C3,4,9S showed a similar binding to CaM, and Ng-IQless and Ng-I33Q did not bind CaM. Ng-S36D showed an intermediate level of CaM binding. Results are means \pm SEM of four independent experiments.

Figure 6.- Effect of PLD overexpression on Ng subcellular distribution

NIH-3T3 cells were transfected with Ng and either PLD1-GFP (**Upper panel**) or PLD2-GFP (**Lower panel**) and analyzed using the cool IF method before (upper row) or after (lower row) 60 sec of “in situ” permeabilization with Saponin. Note that Ng labelling at the plasma membrane is restricted to a peripheral patch that showed strong fluorescence. After saponin extraction, Ng is visualized in perinuclear (PLD1) or dispersed cytoplasmic (PLD2) vesicles. These vesicles were bigger than those observed without PLD overexpression. No colocalization between Ng and with PLDs was observed.

Figure 7.- Effect of PtdIns(4,5)P₂ availability on Ng subcellular distribution

(**Upper panel**) NIH-3T3 cells were transfected with Ng and PIP5KI α and their distribution analyzed using the cool IF method. In both motile (upper row) and quiescent (lower row) cells Ng showed a predominant distribution at the plasma membrane characterized by its presence in multiple small protrusions or blebs that displayed some colocalization with PIP5KI α . (**Lower panel**) NIH-3T3 cells were transfected with Ng, PIP5KI α and PLD2-HA and their distribution analyzed as described above. The expression of PIP5KI α also alters PLD2-HA localization at the plasma membrane. PIP5KI α , PLD2-HA and Ng show a typical spotty distribution characterized by their presence in multiple small protrusions or blebs at the cell periphery. Outlines in the upper row are shown magnified in the lower row. Colocalization among PIP5KI α , PLD2-HA and Ng can be observed at the protrusions.

Figure 8.- Model depicting Ng participation in postsynaptic signal transduction.

Under resting conditions (low Ca²⁺, low PKC activity), Ng locally traps free CaM and PA. Following glutamate receptor activation (NMDA-R and mGluR), both CaM and PA are released and participate in signal transduction pathways that favour LTP over LTD. (AC: Adenylyl Cyclase; NOS: Nitric Oxide Synthetase; CaMK: Ca²⁺/CaM-dependent protein kinase; PLC: Phospholipase C; DAG: Diacyl glycerol; PP1c: Protein Phosphatase 1 catalytic subunit; mTOR: mammalian Target of Rapamycin).

Figure 1

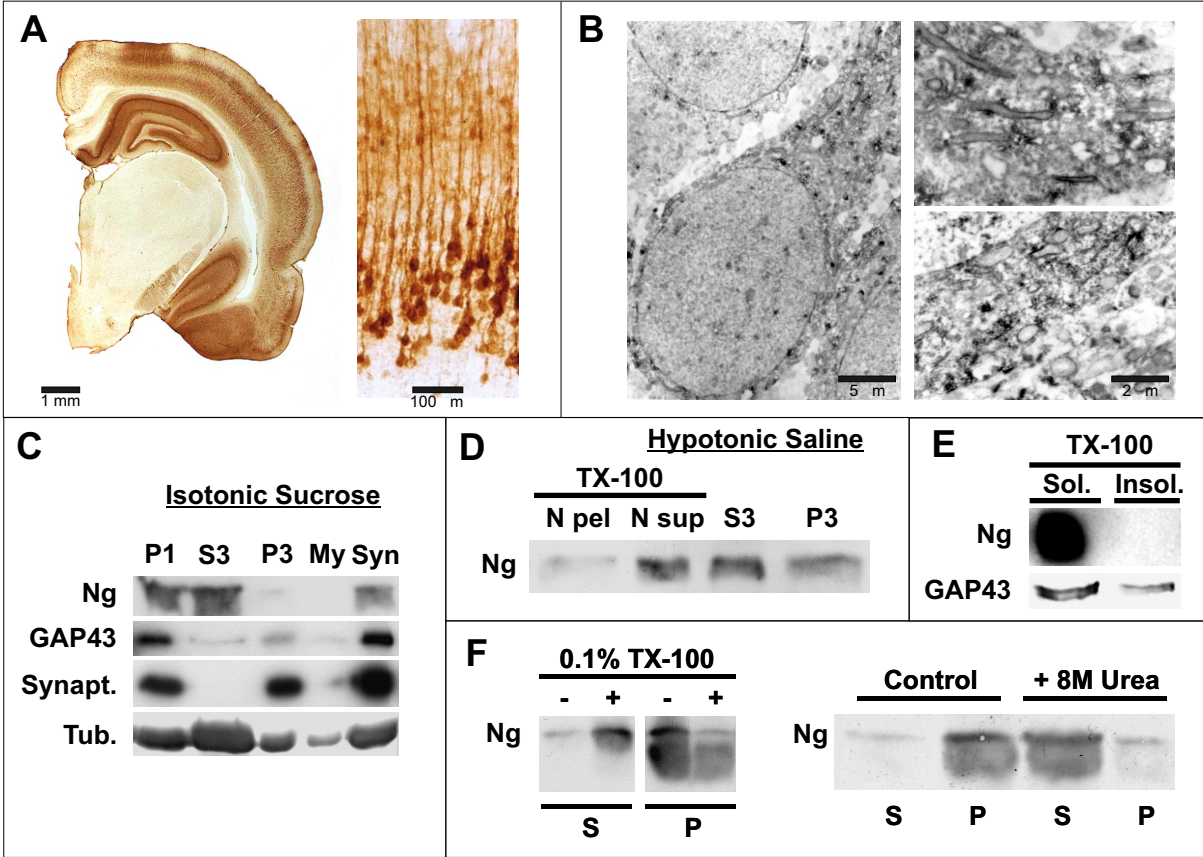


Figure 2

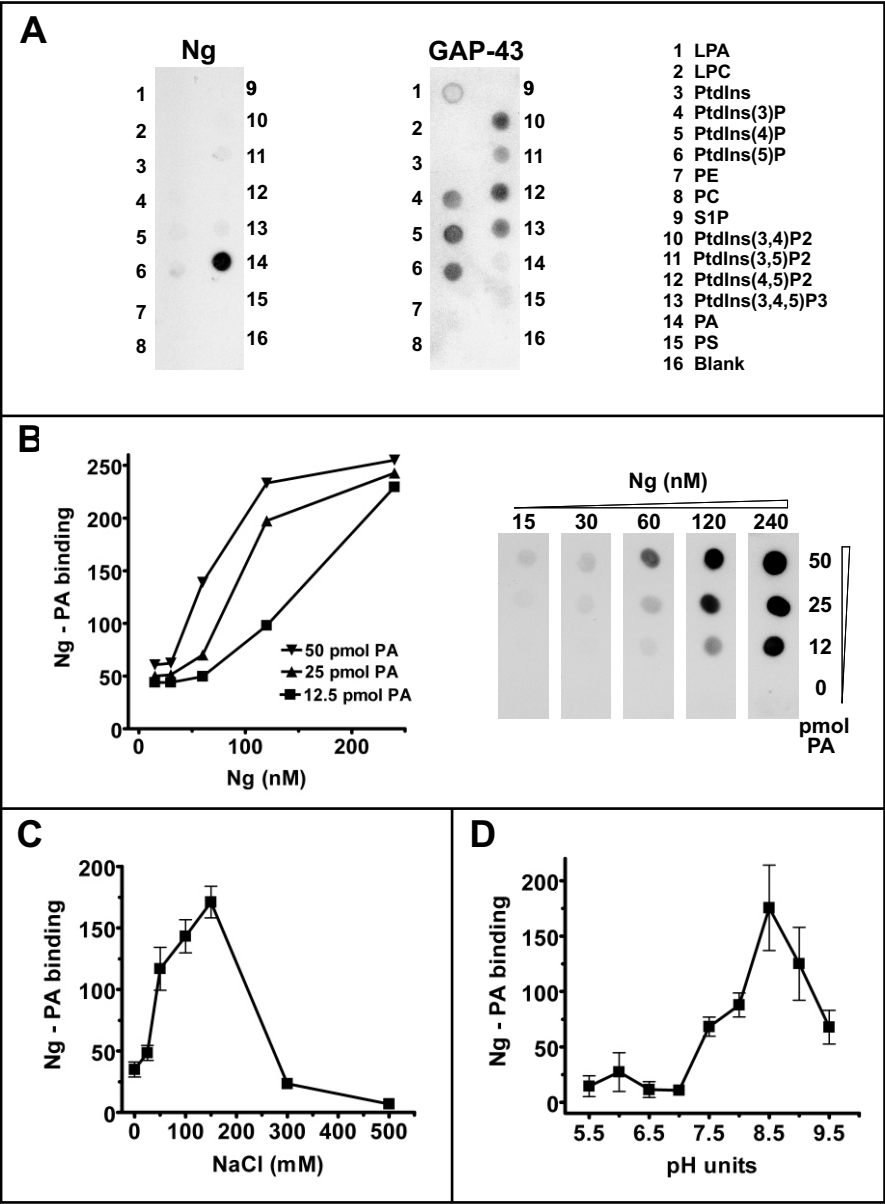


Figure 3

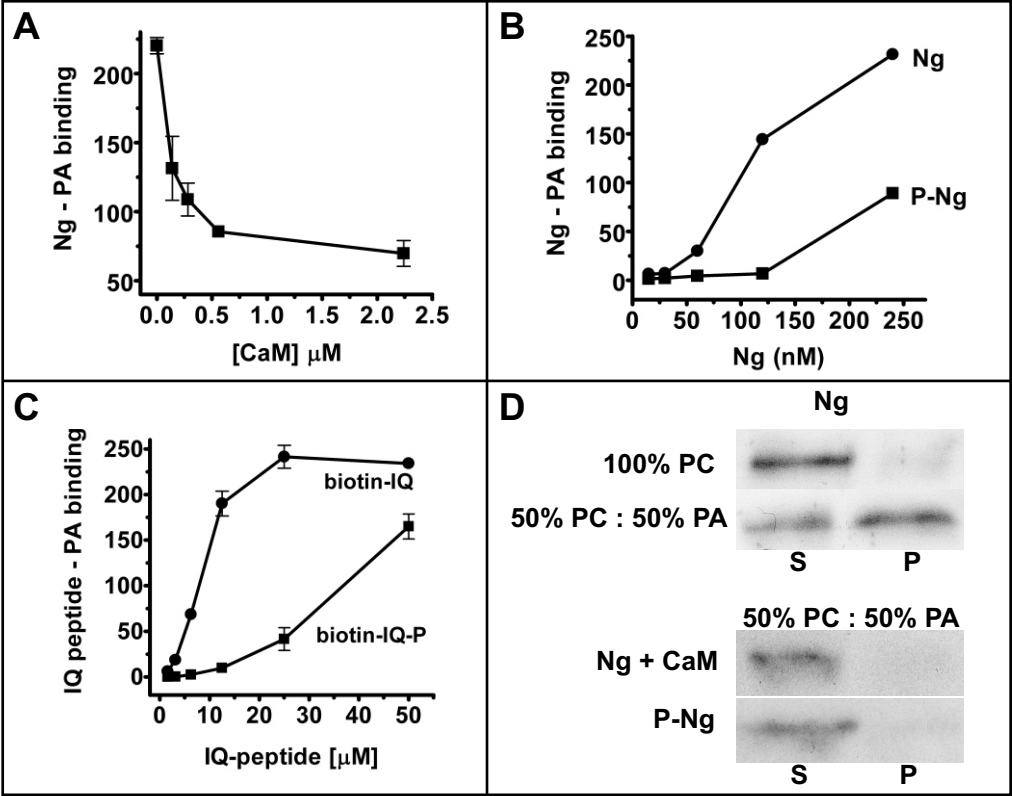


Figure 4

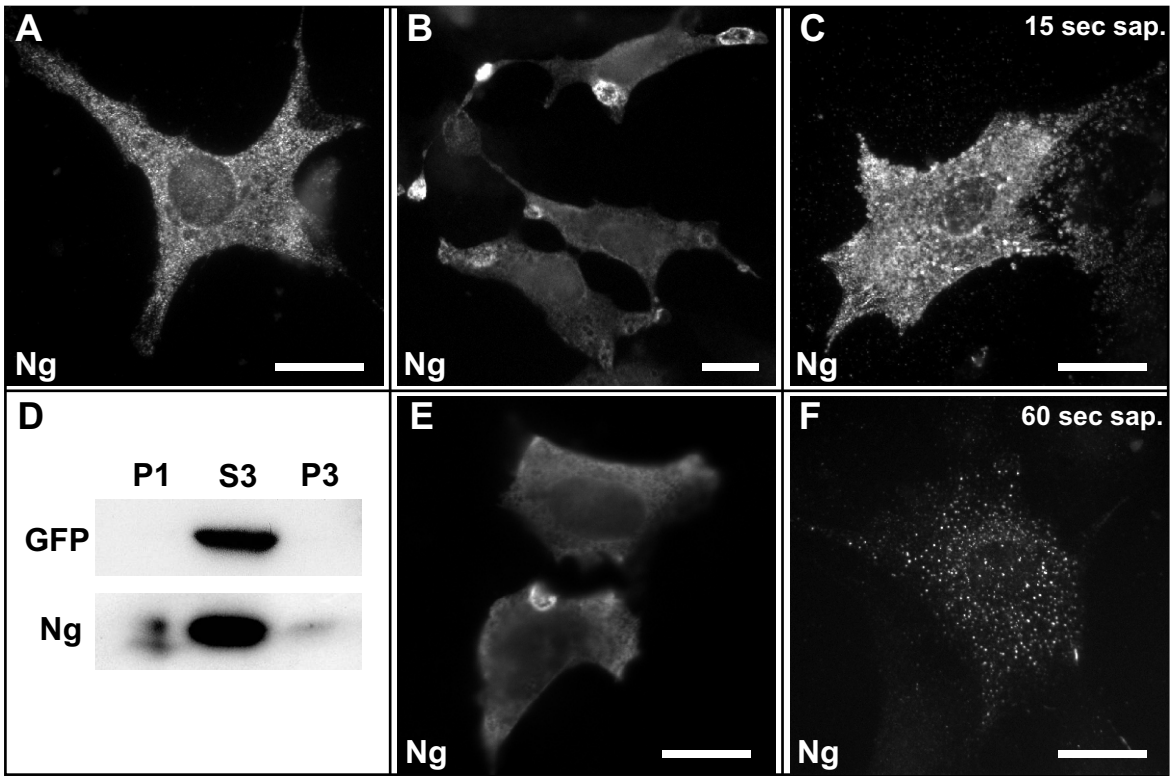


Figure 5

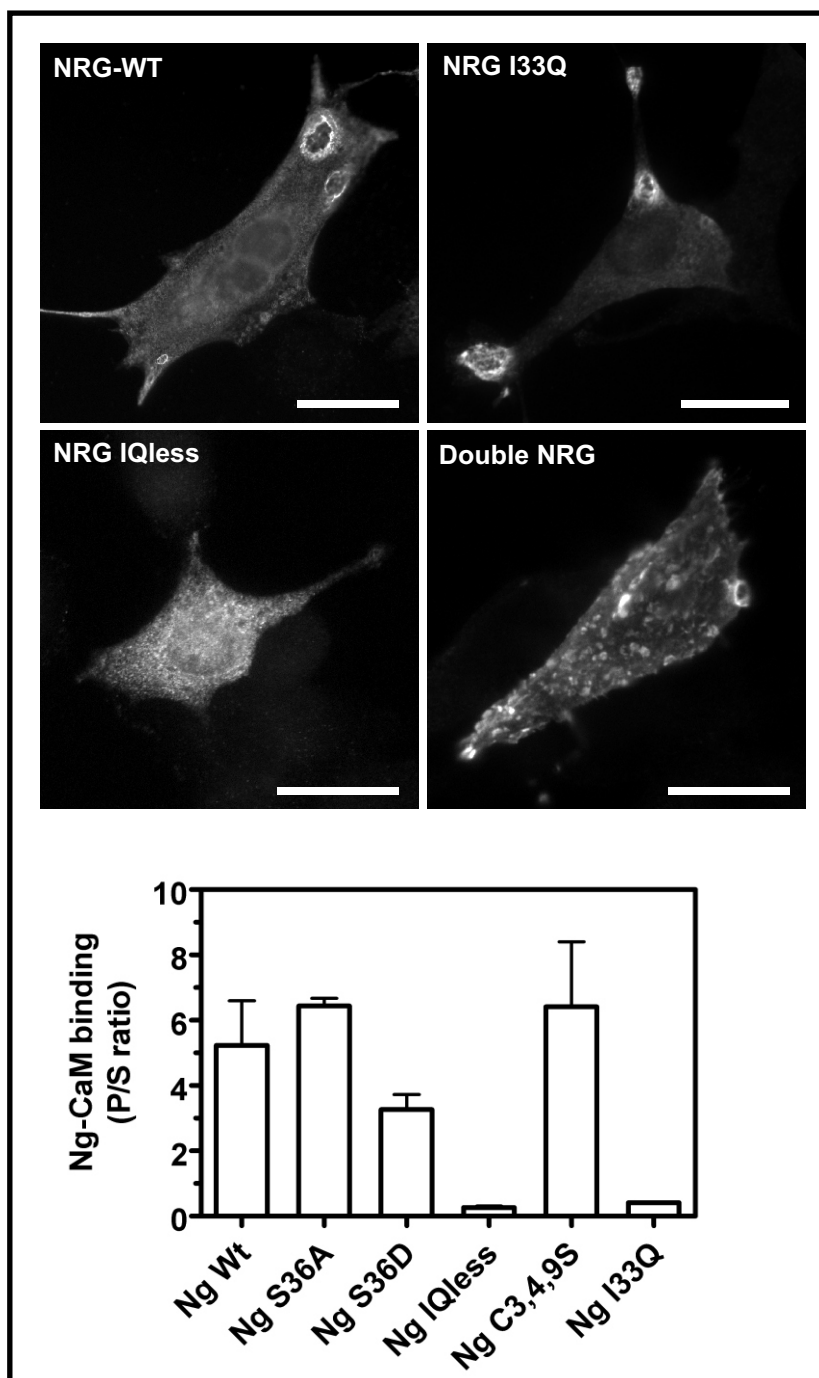


Figure 6

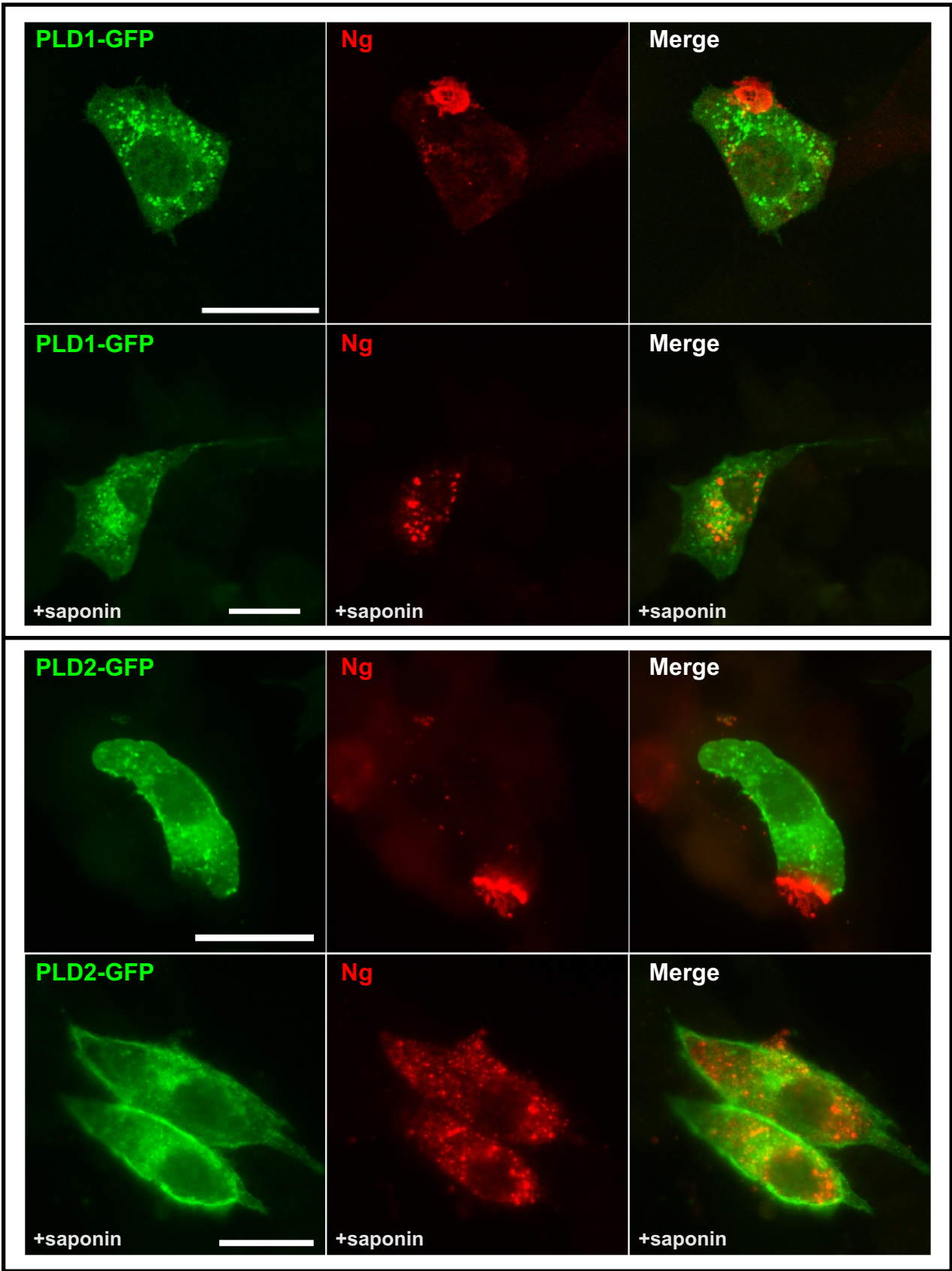


Figure 7

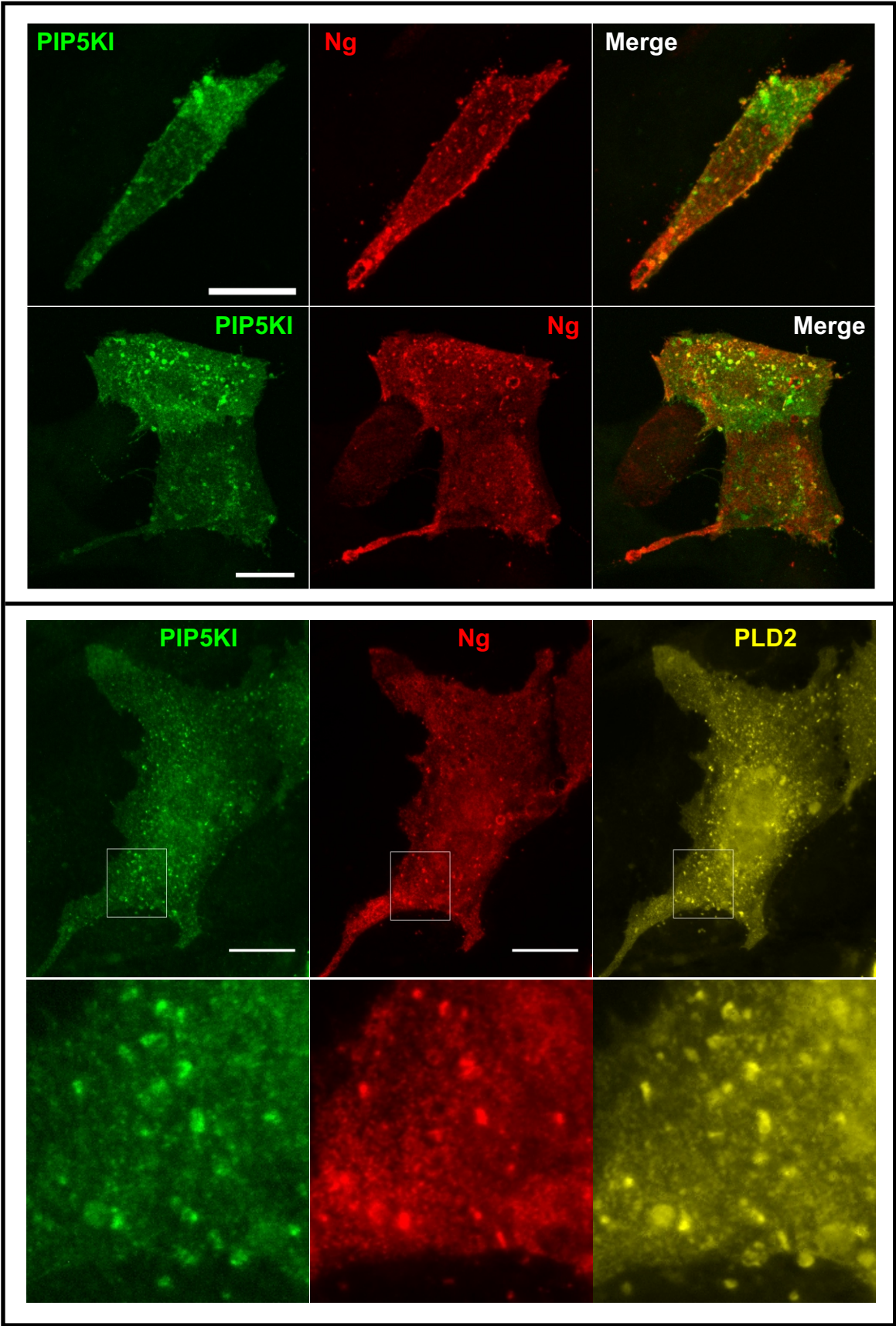


Figure 8

