Differential and Regulated Binding of cAMP-dependent Protein Kinase and Protein Kinase C Isoenzymes to Gravin in Human Model Neurons

EVIDENCE THAT GRAVIN PROVIDES A DYNAMIC PLATFORM FOR THE LOCALIZATION OF KINASES DURING NEURONAL DEVELOPMENT

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The membrane cortex has an important role in generating and maintaining spatially and functionally distinct domains in neurons. As a tool to functionally characterize molecules of the membrane cortex, we generated novel monoclonal antibodies against a fraction enriched for components of the neuronal membrane skeleton. We obtained two antibodies against the kinase-anchoring protein gravin. Gravin was strongly up-regulated during differentiation of human model neurons (NT2-N neurons) and was enriched at the inner peripheral cortex in close proximity to the plasma membrane where its localization primarily depended on association with membranes. In differentiated neurons, gravin colocalized in putative signaling complexes with protein kinase C (PKC) and partially with PKA and cAMP-dependent protein kinase (PKA). Colocalization with PKC was not observed. PKC, PKC, and PKA but not PKC coprecipitated with gravin indicating physical interaction. Binding of gravin to PKC required the presence of Ca2+ and was increased after inhibition of PKC. In contrast, binding of PKC and PKA were independent of Ca2+ and PKC inhibition. Activation of PKC decreased binding of PKA to gravin, decreased its association with the plasma membrane, and reduced the mean size of gravin particles. Taken together the data suggest that gravin provides a dynamic platform to localize kinases in an isoenzyme-specific and activation-dependent manner at specific sites in neurons.

During development, many neurons establish a complex morphology that is characterized by the formation of multiple and in many cases highly branched neurites. Within the neurites, many organelles and proteins exhibit a nonuniform distribution that is thought to provide the basis for the formation of structurally and functionally distinct domains. For example, mitochondria are clustered at sites of high energy consumption, and several cytoskeleton-associated proteins are enriched at positions where active process outgrowth occurs. Some kinases and phosphatases also show a nonuniform distribution in neurons and are enriched at specific regions. In many cases this is achieved by locally anchoring them to cytosolic filament systems or to components of the cortical membrane skeleton.

It is thought that proteins such as protein kinase A anchoring proteins (AKAPs) and several classes of protein kinase C targeting proteins serve as linker proteins to position certain kinases to these immobile structures in a dynamic and region-specific manner. AKAPs are a group of structurally unrelated proteins that are able to target PKA to specific organelles or subcellular structures (for reviews see Refs. 1 and 2). Protein kinase C-targeting proteins such as substrates interacting with C kinase and receptors of activated C kinase similarly localize PKC (3). Recently, multivalent scaffolding proteins that bind several kinases and phosphatases have been described. Examples are the neuronal AKAP-79 that is enriched in post synaptic densities and binds PKA, PKC, and PP2B through distinct domains (4, 5), and the protein gravin, which was originally identified as an antigen of autoantibodies in patients with myasthenia gravis (6). Gravin is a myristoylated high molecular weight protein that is expressed in several tissues and highly expressed in brain (7). In vitro, gravin expression correlates with cell adhesion and migration (8, 9) suggesting a role of gravin also during neuronal development where such mechanisms have an important role. Like AKAP-79, gravin binds PKA and PKC through distinct domains (8) and may be involved in localizing kinases to specific domains in a cell.

The first 1000 amino acids of gravin are 69% identical to the respective part of rodent SsCKS ("Src-suppressed C kinase substrate") that was originally identified as a negative mitotic regulator (10). However, because the sequence similarity in the remaining ~800 amino acids at the carboxyl terminus is very low (<20%), it is not clear whether the two proteins are orthologous or homologous proteins. SsCKS is a major in vitro substrate of PKC and is associated with the cortical cytoskeleton and enriched in plasma membrane and podosomes (11). Like gravin, SsCKS is myristoylated and contains several potential PKC- and one PKA-binding motif. Overexpression of

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¶ The abbreviations used are: AKAPs, protein kinase A anchoring proteins; PKC, protein kinase C; PKA, CAMP-dependent protein kinase; AF, area fractions; MALDI-MS, matrix-assisted laser desorption ionization-mass spectrometry; BSA, bovine serum albumin; PBS, phosphate-buffered saline; RT, room temperature; PMSF, phenylmethylsulfonyl fluoride; DOC, deoxycholate; PNS, post-nuclear supernatant; DMEM, Dulbecco’s modified Eagle’s medium; FCS, fetal calf serum; PS, phosphatidylycerine; PMA, phorbol myristate acetate; BIM, bisindolylmaleimide; PP1, 1,4-piperazinediethanesulfonic acid; SsCKS, Src-suppressed C kinase substrate; TFR, transferrin receptor.
SSeCKS in cultured cells leads to cell flattening, formation of filopodial and lamellipodial-like extensions, and a loss of stress fibers (12). Inhibition of SSeCKS expression in turn increases the formation of stress fibers (13). Evidence exists that phosphorylation by PKC affects the intracellular distribution of SSeCKS, and phosphorylated SSeCKS is concentrated in membrane protrusions and ruffles (14). How gravin is distributed in neurons and whether similar mechanisms operate to localize gravin is not known.

Both PKA and PKC are highly expressed in brain and are important for neuronal function, e.g., learning and memory (for reviews see Refs. 15 and 16). Treatment of hippocampal neurons with PKA- or PKC-specific activators or inhibitors affects neurite outgrowth and branching (17, 18). PKA exists as an inactive tetramer composed of two regulatory and two catalytic subunits. Intracellular CAMP binds to the regulatory subunits, which causes dissociation of the complex and makes the enzyme catalytically active. Multiple forms of regulatory and catalytic subunits exist of which the β-forms are enriched in brain. Most AKAPs bind only the regulatory subunit II. The family of PKC isoenzymes consists of monomeric proteins that can be divided into three groups based on structural differences, the conventional, novel, and atypical PKCs (19). Conventional PKCs (α, β, and γ) can be activated by membrane phospholipids, Ca2+, and diacylglycerols and appear to have similar substrate specificities. Novel PKC isoenzymes (δ, ε, θ, and η) are independent of Ca2+, and atypical PKCs (ζ, η, and Λ) are independent of Ca2+ and diacylglycerols. How the different PKC isofoms are localized in neurons is largely unknown.

To analyze the role of gravin in localizing kinases in human neurons, a well established model for terminally differentiated, postmitotic, and polar central nervous system neurons (NT2-N neurons, a well established model for terminally differentiated, NT2-N cells from two 10-cm tissue culture dishes were counted, and the submembranous cortex. The unbound fraction was centrifuged for 30 min at 100,000g to separate the cytosol (supernatant) from uncracked membranes.

For detergent extraction, NT2-N cells from one 10-cm tissue culture dish were washed twice with PBS, scoured off the coverslip by sonication buffer (80 mM PIPES/KOH, pH 6.8, 1 mM MgCl2, 1 mM EGTA, 30% (v/v) glycerol), counted and collected for 5 min at 37°C, and centrifuged for 5 min at 5000 x g. The pellet was resuspended in 150 µl of extraction buffer containing either 60 µg/ml digitonin or 1% (v/v) Triton X-100 and 10 µg/ml each of leupeptin and pepstatin, 1 mM PMSF, and protease/inhibitor mixture (Roche Applied Science), homogenized by 15 strokes with a tight-fitting steel homogenizer, and centrifuged for 5 min at 13000 x g, and the supernatant (lysate) was collected. Protein concentrations were determined using the method of Bradford (25) with BSA samples containing 20% RIPA as standards.

Triton X-100 lysates were prepared the same way except that Triton buffer (1% (v/v) Triton X-100, 150 mM NaCl, 50 mM Tris/ HCl, pH 8.0) was used instead of RIPA. For fractionation by differential centrifugation, NT2-N cells from two 10-cm tissue culture dishes were washed twice with PBS, sonicated off the coverslip by sonication buffer (80 mM PIPES/KOH, pH 6.8, 1 mM MgCl2, 1 mM EGTA, 30% (v/v) glycerol), counted and collected for 5 min at 37°C, and centrifuged for 5 min at 5000 x g. The pellet was resuspended in 150 µl of RIPA buffer. Plasma membrane fractions were prepared by microsphere separation of surface-biotinylated cells essentially as described previously (23). Briefly, two 10-cm tissue culture dishes of NT2-N cells were surface-biotinylated with a cleavable S-S biotin derivative, incubated with streptavidin-coated microspheres, and cross-linked (26). The homogenate was separated using a magnetic bead attractor into an unbound fraction and a fraction enriched for plasma membranes and the submembranous cortex. The unbound fraction was centrifuged at 100,000 x g to separate the cytosol (supernatant) from uncracked cells and organelles. The cytosol fraction was concentrated by trichloroacetic acid/DODC precipitation (26). The bound fraction was washed three times, separated from the beads by cleavage of the S-S bridge of the biotin derivative, and pelleted at 100,000 x g to yield a fraction enriched for plasma membranes and components of the membrane skeleton.

Apoptotic Purification of Cellular Lysates and MALDI-MS Analysis—1 µg of anti-gravin antibody (JF-74) was covalently coupled to 500 µl of protein A-Sepharose beads (Amersham Biosciences) using dimethyl-pimelimidate according to standard procedures (27) and packed into a column for chromatography. Triton X-100 lysate from 3 x 10^7 differently transfected SK-N-BE (2) cells was loaded, and the column was washed with...
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5 ml of RIPA buffer and eluted with 1 ml of 100 mM glycine, pH 1.8. The eluate was lyophilized, resuspended in SDS sample buffer, and separated by SDS-PAGE on 5% acrylamide. Half of the gel was subjected to immunoblotting with JP-74 antibody, and the other half was stained with Coomassie Blue. The Coomassie-stained band that corresponded to the band detected by immunoblot was excised, repeatedly washed with water and water/acetonitrile (1:1), and digested overnight with 19 μg/ml trypsin at 37°C. The peptides were analyzed by matrix-assisted laser desorption ionization-mass spectrometry (MALDI-MS) at the protein analysis service unit of the DKFZ (Heidelberg, Germany). Samples were prepared using thin film preparation techniques, and MALDI mass spectra were recorded as described previously (28). Data base search was conducted with the peptide masses against the nonredundant data base of the National Center for Biotechnology Information with the program ProFound (wwwBird1.192/192/192.profound.

Identification of a Human Neuronal Cortical Membrane Skeleton Antigen as Gravin—In an immunological approach to obtain novel antibodies against components of the neuronal membrane skeleton, a fraction was prepared that was enriched in inner peripheral plasma membrane proteins using microsphere separation of homogenized human model neurons (Fig. 1A). Previously we have shown that this procedure yields a protein mixture that contains components of the submembranous actin cortex but is largely devoid of cytosolic proteins (23). The isolated material was used to immunize mice and to produce hybridoma lines. Screening by immunofluorescence and immunoblot analysis resulted in the isolation of a panel of clonal hybridoma lines that produced antibodies against corti-

cell separation of homogenized human model neurons (Fig. 1A). The data indicate that we have generated two novel monoclonal antibodies that specifically detect gravin in human neurons by immunocytochemistry and immunoblot analysis, which can be used to analyze gravin localization and function in neurons.

Gravin Is Up-regulated during the Differentiation of Human Model Neurons and Associates with the Membrane Cortex—It has been reported previously that gravin is expressed in different cell types (6, 7). To determine whether gravin is also expressed in neuronal precursor cells and whether neuronal differentiation affects the expression level, the NT2/NT2-N system was employed. NT2 cells are a human teratocarcinoma cell line that shows characteristics of central nervous system neuronal progenitor cells and can be differentiated in vitro to yield terminally differentiated polar and postmitotic neurons.

for 30 min at RT. The suspension was passed five times trough a 27-gauge needle, incubated on ice for 5 min, and centrifuged for 5 min at 13,000 × g and 4°C. The supernatant was collected and centrifuged again. The remaining supernatant was incubated with 5 μg/ml JP-74 monoclonal IgG or, as a control, with 5 μg/ml of an unrelated monoclonal IgG for 90 min at 4°C. 30 μl of protein A beads were added (washed 3 times in a master mix of protein A beads was observed. Immunoprecipitation with a gravin antiserum (kind gift of Dr. J. D. Scott) confirmed that JP74 recognized gravin and that also the other antibody (JP98) recognized the same protein (Fig. 1C). Furthermore, both antibodies were immunoreactive with cells transfected with human gravin cDNA (kind gift of Dr. J. D. Scott) (Fig. 1D). The data indicate that we have generated two novel monoclonal antibodies that specifically detect gravin in human neurons by immunocytochemistry and immunoblot analysis, which can be used to analyze gravin localization and function in neurons.

immunoprecipitations were performed with RIPA lysates according to standard procedures (27). For immunoprecipitation experiments, 3 × 10⁶ (NT2-N) or 5 × 10⁶ (SK-

To confirm the results, the sequence of one of the peptides was also determined by MALDI post-source decay (29).

immunoprecipitation with a gravin antiserum (clone JP-98, if not stated otherwise), anti-tubulin (DM1A), anti-actin (Amersham Biosciences), and anti-transferrin receptor (H68.4, WAK-Chemie, Bad Soden) antibody, or polyclonal anti-gravin (J. D. Scott, Portland, OR), anti-NF-M antiserum (Chemicon), anti-PRC2, anti-PRC1, and anti-PRA antimserum (all from Santa Cruz Biotechnology) were used. As secondary antibodies, horseradish peroxidase-coupled goat anti-mouse (Jackson Immunoresearch, West Grove, PA) and horseradish peroxidase-coupled mouse anti-rabbit (Sigma) antiserum was used. Detection used enhanced chemiluminescence (ECL) (Amersham Biosciences) and was performed according to the manufacturer’s protocol. Quantification of the blots was carried out with an Arcus II scanner (Agfa-Gevaert) and the program NIH Image 1.61/ppc.

Antibodies—The isolated material was used to immunize mice and to produce hybridoma lines. Screening by immunofluorescence and immunoblot analysis resulted in the isolation of a panel of clonal hybridoma lines that produced antibodies against cortically enriched antigens. Two of the antibodies detected a protein with an apparent molecular mass of ~300 kDa which showed a very similar subcellular distribution. The antigen of one antibody (JP74) was affinity-purified and subjected to MALDI-MS after tryptic digest (Fig. 1B). By data base search, 23 of the detected peptides matched the kinase-anchoring protein gravin and covered 24% of the protein sequence corresponding to a probability of 99% (program ProFound) for the analyzed protein to be gravin. No other notable protein match was observed. Immunoprecipitation with a gravin antiserum (kind gift of Dr. J. D. Scott) confirmed that JP74 recognized gravin and that also the other antibody (JP98) recognized the same protein (Fig. 1C). Furthermore, both antibodies were immunoreactive with cells transfected with human gravin cDNA (kind gift of Dr. J. D. Scott) (Fig. 1D). The data indicate that we have generated two novel monoclonal antibodies that specifically detect gravin in human neurons by immunocytochemistry and immunoblot analysis, which can be used to analyze gravin localization and function in neurons.
In NT2 cells, gravin is expressed at a low level (Fig. 2A, day 0). During neuronal differentiation, gravin expression strongly increases and remains at high level at later time points (Fig. 2B, 21 days). Interestingly, the up-regulation precedes the expression of the neuronal marker protein NF-M indicating that it occurs early during the development of neurons.

The antibodies against gravin were obtained after immunization with a neuronal membrane skeleton fraction suggesting that gravin is enriched at the membrane cortex. To analyze the association of gravin with the membrane skeleton in more detail, subcellular fractionation of human neurons was performed. Gravin was enriched in the pellet fraction after ultra-centrifugation of the PNS indicating association with either the membrane and/or the cytoskeleton of the cells (Fig. 2B, left). To distinguish between these possibilities, neurons were extracted using different detergents. In the presence of digitonin, which perforates but does not completely solubilize plasma membranes (as indicated by the presence of the integral membrane protein transferrin receptor (TFR) in the pellet fraction), gravin was highly enriched in the cellular pellet (Fig. 2B, middle). In contrast, gravin was exclusively present in the extracted supernatant when membranes were solubilized with Triton X-100 (Fig. 2B, right). This indicates that the subcellular distribution of gravin in neurons depends primarily on its association with membranes rather than on its interaction with cytoskeletal components. In addition, a substantial portion of gravin was present after microsphere separation in a fraction enriched in plasma membranes and proteins of the membrane cortex suggesting a close association with plasma membrane components (Fig. 2C). Immunelectron microscopy confirmed an enrichment of gravin at the inner peripheral cortex of neurites in close proximity to the plasma membrane (Fig. 2D). Interestingly, many of the gold particles were clustered suggesting a nonuniform distribution of gravin (arrows).
Association of Gravin with Membrane Is Decreased after PKC Activation—To determine whether interaction of gravin with the plasma membrane is regulated in neurons, we tested the effect of activation of kinases on the association of gravin with the membrane fraction. PKC isoenzymes represent potential candidate kinases because they are abundant in neurons and can interact with membranes and gravin (9, 19). In control cells, the majority of gravin (~75%) was found to be present in the pellet fraction after subcellular fractionation of neuronal homogenates (Fig. 3) indicating its association with membranes. Activation of PKC with phorbol ester (PMA) resulted in a significant redistribution of gravin to the supernatant cytosol (<20% remained in the pellet fraction). No changes in the distribution of actin and tubulin were observed, indicating that PMA does not affect the assembly state of the cytoskeleton in our conditions. To determine the time course of the effect of PMA on gravin distribution, cellular fractionation experiments were performed after different incubation times with PMA. After 10 min of PMA treatment, the amount of gravin in the pellet fraction was already decreased to about half of the maximum decrease which was reached after 30 min after incubation (36 and 34% of gravin in the pellet fraction after 30 and 60 min of incubation, respectively; data are expressed relative to a control that has been incubated with the carrier for the same time). The data suggest that, in neurons, the membrane association of gravin dynamically responds to the activity state of PKC.

In Neurons, Gravin and PKCβII Almost Exclusively Colocalize in Putative Signaling Complexes—Because gravin is a multivalent scaffolding protein that interacts with different kinases, it may differentially localize kinases to specific domains in the neuron. To analyze the distribution of gravin and a
potential interaction with different kinases on a cellular level, immunofluorescence microscopy of dissociated, polar neurons was performed. Gravin exhibited a particulate and nonuniform staining pattern that was present in the cell body and neurites (Fig. 4A). The staining was very similar in unextracted and permeabilized or in digitonin-extracted neurons consistent with an association of gravin with the plasma membrane and the actin cortex. In neurites, gravin-containing complexes appeared to be concentrated close to branch points. Gravin was present in both axons and dendrites as confirmed by double immunofluorescence with the axon-specific marker protein tau (data not shown). To determine the size of gravin particles in neuronal processes, morphometric image analysis was performed on neurons that had been immunostained against gravin. The mean area per gravin particle in neuronal processes corresponded to about 0.27 \( \mu \text{m}^2 \) (S.E. \( \pm 0.05, n = 10 \) microscopic frames). Treatment with PMA reduced the mean size of the particles by about 70% after 1 h of incubation (Fig. 4B). After 10 min of incubation, a significant decrease of the mean area of gravin particles with time of incubation (Fig. 4A).

For every experimental condition, 10 microscopic frames were selected and analyzed. Means \( \pm \) S.E. are shown.

It is known that gravin not only interacts with PKC isoenzymes but also with PKA. PKA consists of different subunits of which the regulatory subunit II (RII) has been reported previously to bind to gravin (8). The catalytic subunit \( \beta \) is highly expressed in neurons. The PKA catalytic subunit \( \beta \) showed a uniform distribution in neurons similar to the PKCo distribution (Fig. 5D). However, in many cases, stainings for gravin and the catalytic subunit \( \beta \) appeared mutually exclusive in neurites as evident in the images with higher magnification (Fig. 5E). A very similar distribution was observed for RII where many gravin complexes in the neurites did not stain for RII and vice versa (Fig. 5F).

Taken together, the immunocytochemical data suggest that gravin differentially interacts with PKA and PKC isoenzymes in neurons. In particular, gravin and PKC\(\beta\)I\(\II\) show a remarkable colocalization in particulate structures in the cell body and neurites which may represent locally organized signaling complexes.

**PKC Differentially Binds to Gravin in an Isoenzyme, \( \text{Ca}^{2+} \)-and Activation-dependent Manner**—In order to analyze the interaction of gravin with kinases by biochemical means, we first analyzed the expression pattern of gravin and PKA and PKC isoenzymes in NT2 neuronal precursor-like cells, NT2-N neurons, and differentiated SK-N-BE (2) human neuroblastoma cells. Gravin and all PKC and PKA isoenzymes tested with the exception of PKCo were considerably up-regulated in
NT2-N neurons compared with the precursor cell line (NT2 cells) (Fig. 6A). Also in SK-N-BE (2) cells, gravin was strongly expressed similar to NT2-N neurons. However, both cell types differed in the expression of PKC isoenzymes. Whereas PKC$_{\alpha}$/H9251 and PKC$_{\epsilon}$/H9280 was substantially higher expressed in SK-N-BE (2) cells, PKC$_{\epsilon}$/H9252 expression was much more prominent in NT2-N neurons. No major difference in the expression of PKA cat was observed.

To analyze the binding of the kinases to gravin and its potential regulation, coimmunoprecipitation experiments with lysates from NT2-N neurons and differentiated SK-N-BE (2) cells were performed. The $\beta$-catalytic PKA subunit and the conventional PKC isoenzymes $\alpha$ and $\beta$II coprecipitated with gravin (Fig. 6B). In contrast, no interaction was detected with the novel PKC isoenzyme $\epsilon$, although it was clearly expressed in the neurons (Fig. 6A), consistent with the immunofluorescence data, where PKC$_{\epsilon}$ was the only isoform and where almost no overlapping staining with gravin was observed (refer to Fig. 5C).

Because phosphatidylserine (PS) and Ca$^{2+}$ are cofactors for the activity of PKC, they may also be involved in the binding of PKC to anchoring proteins (11, 33). However, addition of exogenous PS did not affect the amount of coprecipitated kinases (Fig. 6C), although it has been reported previously that PS is essential for binding of PKC to recombinant gravin fragments (8). This may indicate that, in our experiments, the amount of endogenous PS was sufficient to allow binding of PKC to gravin. Interestingly, depletion of Ca$^{2+}$ in the lysate by addition of EGTA resulted in an almost complete loss of PKC$_{\alpha}$ binding but did not affect the interaction of PKC$\beta$II and PKA with gravin. Thus, the two closely related conventional PKC isoenzymes $\alpha$ and $\beta$II drastically differ in their property to interact with gravin which provides a cellular mechanism to regulate the binding of PKC to gravin in an isoenzyme-specific manner.

To determine whether the activation state of kinases affects their interaction with gravin, NT2-N neurons were incubated with the broad spectrum kinase inhibitor staurosporine. Interestingly, staurosporine treatment resulted in a strong increase in the binding of PKC$_{\alpha}$/H9251 to gravin but not of any other kinase (Fig. 6D). Also incubation with the PKC-specific inhibitor BIM significantly increased the binding of PKC$_{\alpha}$/H9251 by a factor of more than 3 (Fig. 6E and F). Activation of PKC with PMA in turn decreased the binding of PKC$_{\epsilon}$/H9251 to less than 40%. Again, no significant change was observed for the other kinases. Thus, similar to Ca$^{2+}$, inhibition or activation of PKC specifically affects the binding of PKC$_{\alpha}$ to gravin but not of PKC$\beta$II or PKA, further indicating that gravin interacts with PKC in an
isoenzyme-specific manner. The results were very similar for SK-N-NE (2) cells except for PKCα where only a very weak binding to gravin was observed in untreated cells. However, also in these cells, binding of PKCα to gravin strongly increased after inhibition of PKC with BIM and depended on Ca²⁺, indicating that differential binding of the kinases to gravin is a general feature of human neuronal cells rather than a singularity of the NT2-N model system (Fig. 6G).

To determine whether inhibiting PKC results also in a change in the amount of colocalization with gravin, morphometric image analysis of double-immunofluorescence micrographs was performed. Treatment with BIM resulted in an about 50% increase of the amount of PKCα colocalizing with gravin (52 ± 6% and 32 ± 10% with BIM and carrier, respectively; 10 microscopic frames were analyzed per experiment) indicating that the increased interaction after coimmunoprecipitation is reflected by an increase in colocalization. In similar experiments, the effects of treatment with the calcium ionophore ionomycin (10 μM), glutamate (50 μM in Krebs buffer) that stimulates ionotropic and metabotropic glutamate receptors present in NT2-N neurons (34), or the β-adrenergic receptor agonist isoproterenol (10 μM) that activates this prototypic G-protein-coupled receptor coupled to adenyllyclase (35, 36) were tested. In contrast to the treatment with BIM, no reproducible effect of any of these drugs on the amount of colocalization of PKCα with gravin was observed after morphometric image analysis. Also in coimmunoprecipitation experiments, stimulation of NT2-N neurons with isoproterenol did not affect binding of PKCα with gravin (Fig. 6H).

**DISCUSSION**

The cortical membrane skeleton has an important role in the development of the characteristic morphology and function of neurons. In an immunological approach we generated two novel monoclonal antibodies against gravin as a component of the membrane cortex of human neurons. By using subcellular
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fractionation, detergent extraction, and immunoelectron microscopy, we demonstrated that the majority of gravin is associated with membranes and enriched at the inner peripheral cortex of neurons in close proximity to the plasma membrane similar to its distribution in non-neuronal cells (9, 35). Interestingly, we could show that the association of gravin with neuronal membranes is substantially decreased after treatment with phorbol ester indicating that gravin can switch between a membrane-associated and a soluble form dependent on the activity state of PKC. The primary sequence of gravin has revealed the presence of a myristoylation signal at the amino terminus and several polybasic regions, which could be sufficient to localize gravin to the plasma membrane (8, 37). According to primary sequence analysis by the program “scanprosite” (www.expasy.ch) gravin contains 32 theoretical PKC phosphorylation sites, and phosphorylation may liberate gravin from the membrane by a myristoyl-electrostatic switch similar to what was observed for the PKC substrate myristoylated alanine-rich C kinase substrate, which also contains a myristoyl residue and a polybasic region (38). It should, however, be noted that two different isoforms of gravin have been described that differ in their amino-terminal sequence and that only one contains a myristoylation signal (8, 39). PCR data using primer combinations to detect gravin isoforms with the myristoylation signal indicate the expression of this isoform in NT2-N neurons.2

By using blot-overlay assays it has been shown previously that gravin can interact with PKA and a mixture of conventional PKC isoenzymes (8). In endothelial cells, PKA and PKCα could be coprecipitated with an anti-gravin antibody (9); in blood cells, only PKA coprecipitated with an anti-gravin antisem (8). Here we demonstrate that, in neurons, gravin interacts with PKA and the conventional PKC isoenzymes α and βII but not with PKCε as analyzed by coimmunoprecipitation experiments. By using immunocytochemistry, we show that gravin partially colocalizes with PKA and PKCα but almost completely colocalizes with PKCβII in particulate and nonuniformly distributed structures in the soma, axonal, and dendritic processes. The resistance to detergent extraction indicates tight complexes containing both gravin and specifically anchored kinases. The data suggest that gravin preferentially localizes PKCβII to putative signaling complexes close to the plasma membrane. In another cell type (epidermoid carcinoma cells), dynamic signaling complexes containing gravin, βII-adrenergic receptor, PKA, PKC, PP2B, and some other components have been described (35, 40). In this system, the association of gravin with βIII-adrenergic receptor was increased after agonist stimulation (10 μM isoproterenol) concomitant with an increased association of the receptor-gravin complex with protein kinases A and C (40). In the NT2-N system, isoproterenol treatment at similar conditions did not affect binding of PKCα, PKCβIII, or PKA to gravin as judged by coimmunoprecipitation experiments or analysis of colocalization. It will be interesting to identify other components of gravin-containing signaling complexes in neurons and their involvement during neuronal development.

Gravin acts as an anchoring scaffold for PKA and PKC, and there is evidence that gravin-bound kinases are inactive. In the case of PKA, gravin, like other AKAPs, binds the inactive holoenzyme (1). For PKC it has been demonstrated that a PKC-binding gravin fragment inhibits the activity of PKCβII (8). Thus, regulating the interaction of gravin with kinases may have an important role for the activity state of the kinases at their particular localization. Here we report that the interaction of PKCα with gravin is dramatically increased after inactivation of PKC with BIM and decreased after activation of PKC with PMA. In contrast, the interaction of PKCβII with gravin was not affected by BIM or PMA. In addition, the interaction of PKCα with gravin requires calcium, whereas the binding of PKCβII with gravin was independent of calcium, thus providing a mechanism to regulate the interaction of these conventional PKC isoenzymes with gravin in a neuron.

It is unclear how calcium may affect the binding of PKCα to gravin. In the case of AKAP-79 it has been shown that depletion of calcium does not influence the binding of PKC and that the binding of Ca2+/calmodulin reciprocally affects PKC binding (33). This indicates that the calcium-dependence of the gravin/PKCα and the AKAP-79/PKC interaction are clearly different. Calcium dependence has also been described for the interaction of PKCα with the major neuronal PKC substrate GAP-43 (41). However, PKCα is not a conventional PKC isoenzyme.

During the differentiation of human model neurons, expression of gravin is strongly up-regulated. In parallel, PKA and

Fig. 7. Model for the interaction of gravin with kinases and membranes in neurons and its potential regulation by Ca2+ and the activation state of PKC. In neurons, the majority of PKCβIII but only a minor subpopulation of PKCs and PKA and no PKCε interact with gravin in putative signaling complexes. Activation of PKC by phorbol ester (PMA) decreases the association of gravin with membranes and the binding of PKCs but not of PKCβII or PKA to gravin. In turn, inhibition of PKC by BIM increases binding of PKCα to gravin but not binding of the other kinases. Depletion of Ca2+ leads to a selective loss of binding of PKCα to gravin.

2 C. Bas-Orth and R. Brandt, unpublished observations.
PKC isoenzymes are also up-regulated in these cells. Interestingly, PKCβII, which shows the strongest interaction with gravin, was up-regulated the most. Gravin and PKA and PKC isoenzymes are also expressed in another human neuronal model, SK-N-BE (2) neuroblastoma cells, however at a very different ratio. Nevertheless, the differential interaction and the regulation of the binding of gravin with the kinases were substantially similar. For example, despite a very high expression level of the novel PKC isoenzyme PKCe compared with PKCβII in SK-N-BE (2) cells, only an interaction of PKCβII with gravin was detected by communoprecipitation experiments. This is different when compared with AKAP-79 (another human PKA and PKC anchoring protein present in neurons), which binds both conventional and novel PKC isoenzymes including PKCe (42). The basic features of the localization of gravin and its interaction with kinases as they are evident from our data are summarized in Fig. 7. The model emphasizes the following findings. (i) Gravin anchors a subpopulation of PKA and PKCζ and the majority of PKCβII but no PKCε, in distinct putative signaling complexes close to the plasma membrane. (ii) Activation of PKC by PMA releases the signaling complex to the cytosol, whereas a substantial portion of PKCs is liberated. (iii) Inhibition of PKC by BIM increases the amount of PKCζ that is bound to gravin. (iv) Depletion of Ca2+ leads to a selective loss of the interaction between PKCζ and gravin. Gravin contains three putative binding sites for PKC, and the model shows distinct binding sites for PKCs and PKCβII. It is also possible that both isoforms bind to the same site(s), and our data are consistent with both possibilities. According to the model, gravin may provide a platform to colocalize kinases in an isoenzyme-specific manner with potential substrates at specific sites in a neuron. Upon a local stimulus, specific isoenzymes may be released and/or activated to phosphorylate their adjacent target.

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