Regulation of AKAP-Membrane Interactions by Calcium*

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The AKAP gravin is a scaffold for protein kinases, phosphatases, and adaptor molecules that obligate for resensitization and recycling of β2-adrenergic receptors. Gravin binds to the receptor through well-characterized protein-protein interactions. These interactions are facilitated ~1000-fold when gravin is anchored to the cytoplasmic leaflet of the plasma membrane. Although the N-terminal region (~550 residues) is highly negatively charged and probably natively unfolded, it could anchor gravin to the inner leaflet through hydrophobic insertion of its N-terminal myristate and electrostatic binding of three short positively charged domains (PCDs). Loss of the site of N-myristoylation was found to affect neither AKAP macroscopic localization nor AKAP function. Synthetic peptides corresponding to PCD1–3 bound in vitro to unilamellar phospholipid vesicles with high affinity, a binding reversed by calmodulin in the presence of Ca2+. In vivo gravin localization is regulated by intracellular Ca2+, a function mapping to the N terminus of the protein harboring PCD1, PCD2, and PCD3. Mutation of any of two PCDs eliminates membrane association of the non-myristoylated gravin, the sensitivity to Ca2+/calmodulin, and the ability of this scaffold to catalyze receptor resensitization and recycling.

A-kinase anchoring proteins (AKAP)2 are scaffolds essential for cell signaling (1). Gravin (a.k.a. AKAP250, AKAP12, SSeCKS) is a large AKAP with multivalency for cAMP-dependent kinase (PKA) and C (PKC), phosphoprotein phosphatases (e.g., MARCKS) is a large AKAP with multivalency for cAMP-dependent kinase (PKA) and C (PKC), phosphoprotein phosphatases (e.g., MARCKS) are all capable of binding to membranes and Ca2+/CaM with significant affinity.

We explore herein how the myristate and three N-terminal PCDs contribute to membrane binding, and thus to the function of gravin. Why is membrane binding important? It localizes gravin to the plasma membrane, where it experiences ~1000-fold higher effective concentration of membrane bound β2AR because of the well-established “local concentration” or “reduction of dimensionality” effect (12). Gravin localization is regulated by intracellular Ca2+, a function mapping to the N terminus of the protein harboring PCD1, PCD2, and PCD3. Mutation of any of two PCDs eliminates membrane association of the non-myristoylated gravin, the sensitivity to Ca2+/calmodulin, and the ability of this scaffold to catalyze receptor resensitization and recycling.

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**EXPERIMENTAL PROCEDURES**

HA Tag and CFP Fusion Proteins—The HA-tagged fusion proteins of gravin, N-terminal-truncated HA-gravin, and C-terminal-truncated HA-gravin mutants were constructed as previously described (3) and inserted into an expression vector. To generate HA-tagged short, positively charged calmodulin-binding domains (PCD), the sense primers contained 5'-AR primers encoding the HA tag (YPYDVPDYALV) followed by nucleotides corresponding to 171–177 (for PCD1) and amino acids 297–303 (for PCD2), and amino acids 510–516 (for PCD3). The antisense primes that were synthesized corresponded to amino acids 191–197 (for PCD1) and amino acids 314–320 (for PCD2), and amino acids 531–537 (for PCD3) of human gravin, with the addition of a BamH1 restriction site, following the stop code. The PCR products were cloned into pcDNA3 (Invitrogen) between NheI and BamHI restriction sites.

To generate HA tag gravin in which PCD1 and PCD2 regions were deleted (Gravin ΔPCD1,2), the sense primer contained a 5'-NheI site and nucleotides encoding the HA tag (YPYDVPDYALV) in the front of the N terminus of gravin. The antisense primer corresponding to the sequence of gravin from 488–510 followed by ACC1 site (CCGGTCGACAATATCATTAGC-CTGGAACCTCA). For the second fragment, the sense primer contain the ACC1 site corresponding to the sequence of gravin from membrane to cytoplasm.
DNA sequencing.

When 400 "Cyclone experiments were performed using polymerase chain reactions were performed using Pfu polymerase (Stratagene). The primer sequences, incorporating the assumption that Ca²⁺/CaM and the membrane compete for the peptide (8) in Equation 2,

\[
\frac{[P]_{\text{mem}}}{[P]_{\text{tot}}} = \frac{K[L]_{\text{acc}}}{(1 + K[L]_{\text{acc}} + K_{\text{CaCaM}}[C^{2+}/CaM])}
\]

where \([P]_{\text{mem}}/\[P]_{\text{tot}}\) is fraction of peptide bound to membranes, \(K_{\text{CaCaM}}\) is the association constant of the peptide with Ca²⁺/CaM. Special conditions were required to estimate the

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where \(K_{\text{CaCaM}}\) is the association constant of the peptide with Ca²⁺/CaM. Special conditions were required to estimate the
binding of the PCD1 peptide to membranes and to calmodulin. It is apparent from Fig. 2 that the PCD1 region has fewer basic (blue) and more hydrophobic (green) residues than either the PCD2 or PCD3 regions. Thus a peptide corresponding to PCD1 will have the greatest tendency to aggregate in solution. Although the PCD1 peptide, gravin (171–187), appeared to dissolve in solution by visual inspection, it clearly formed aggregates because this peptide (unlike the PCD2 and PCD3 peptides) was significantly removed from solution by centrifugation. Thus measurements corresponding to Fig. 3A with PCD1 peptide (not shown), and those shown in Fig. 3C were conducted with 0.01% Triton X-100 added to the solutions to solubilize the peptide. Addition of detergent will decrease the affinity of the peptide for the membrane (and probably Ca\(^{2+}\)/CaM), so the affinity values reported in Table 1 for gravin (171–187) should be regarded as underestimates. As a control, we studied the effect of Triton X-100 on the membrane binding of the (apparently soluble) gravin (297–317) peptide. In experiments similar to those illustrated in Fig. 3A with 2:1 PC/PS vesicles, we found that the K value decreased exponentially (binding energy decreased linearly) with the % detergent over the range from 0 to 0.025%. Fortunately, the value extrapolated from the measurements with detergent agreed well with the actual value measured in the absence of detergent for gravin (297–317), suggesting the peptide is present as a monomer in the absence of detergent. The observation that a 2-fold increase in peptide concentration did not affect the value of K is also consistent with the assumption the peptides are binding as monomers to the membrane.

Confocal Microscopy—A431 cells stably expressing gravin-GFP, truncated gravin-CFP, or HA-tagged gravin and propagated on glass slides were either left untreated or stimulated with 10 \(\mu\)M isoproterenol for 30 min, 10 \(\mu\)M A23187 for 30 min, or 20 \(\mu\)M BAPTA-AM for 6 h and then washed twice with Hank’s balanced salt solution, fixed (2% paraformaldehyde, pH 7.2). For immunostaining studies, cells were permeabilized and stained with anti-HA antigen rabbit antibodies (Molecular Probes). Stained objects were imbedded in ProLong (Molecular Probes) anti-fade reagent. Images were acquired on the Zeiss LSM510 microscope using argon and helium-neon lasers (oil-immersion, \(\times 63\) objective). Serial sections were acquired as a Z-stack. Z-stacks of images were exported as TIFF files and processed in Adobe Photoshop 5.5.

FPLC Calmodulin Binding Assay—HA-tagged PCD1, -PCD2, and -PCD3 fragments, each were expressed via transient transfection into A431 cells. Cells were harvested and lysed in a lysis buffer (50 mM Tris-HCl, pH 7.5, 2 mM CaCl\(_2\), 150 mM NaCl, 1% Triton X-100, 0.5% Nonidet P-40, 5 \(\mu\)g/ml aprotonin, 5 \(\mu\)g/ml leupeptin, 100 \(\mu\)g/ml bacitracin, 100 \(\mu\)g/ml benzamidine). Cell lysates were subjected to column chromatography on CaM-immobilized Sepharose-4B matrix. Bound peptides were eluted from the medium by stripping the matrix of Ca\(^{2+}\) using a gradient of EDTA (0–3 mM). The eluted fractions were collected at 1-ml intervals. The eluted proteins were separated by 10–20% SDS-PAGE, transferred electrophoretically to nitrocellulose blots, and the resolved proteins stained by immunoblotting using anti-HA antigen primary antibodies.

Knockdown Studies of Gravin—Antisense morpholino oligonucleotides (morpholinos) were synthesized and purified to cell culture grade (Gene Tools, LLC). Before addition to A431 cells, morpholinos were mixed at a ratio of 1:1 (w/w) with EPEI special delivery solution (Gene Tools, LLC). Cells were treated with morpholinos (5 \(\mu\)g/ml) for 3 days. An additional treatment with morpholinos was performed prior to transfection of the cells with either wild-type or mutant forms of gravin. Following this protocol, cells were analyzed for \(\beta\)-adrenergic agonist-induced (i.e. isoproterenol, 10 \(\mu\)M) desensitization and recovery (i.e. resensitization) after washout of agonist. The suppression of gravin expression was confirmed by SDS-PAGE of whole cell lysates followed by immunoblotting. Similarly, loading controls were established by immunoblotting of actin. Identical treatment of the cultures with “scrambled” sequence morpholinos (designed by the commercial supplier) were employed as a control.

Protocols for Desensitization and Resensitization of \(\beta_2\)AR—Two days prior to the analysis of agonist-induced desensitization, the A431 cells were seeded in 96-well microtiter plates at a density of 25,000–50,000 cells/well. Routinely cells were serum-starved overnight, prior to analysis. Desensitization was accomplished by treating the cells with the \(\beta\)-adrenergic agonist isoproterenol (10 \(\mu\)M) for 30 min. Under these conditions, subsequent \(\beta\)-adrenergic stimulation of cyclic AMP accumulation is severely blunted and the number of cell-surface receptors declines precipitously as the receptors are sequestered and internalized (17). Details of the desensitization protocol are described elsewhere (18).

Analysis of \(\beta_2\)AR Internalization and Recovery of \(\beta_2\)AR to the Cell Membrane—The internalization of \(\beta_2\)AR correlates well with the extent of agonist-induced desensitization. Using the binding of a cell-impermeant, radiolabeled antagonist for the determination of cell surface \(\beta_2\)ARs, the functional status of the recovery of internalized \(\beta_2\)AR could be assayed with great accuracy. Cultures of A431 cells were treated with isoproterenol for 30 min (i.e. “desensitized”) or treated with isoproterenol for 30 min then washed free of agonist for 60 min (i.e. “resensitized”). The cells then were washed with ice-cold phosphate-buffered saline and resuspended in DMEM containing 20 mM HEPES (pH 7.4) and the hydrophilic, membrane-impermeant \(\beta_2\)-adrenergic antagonist \[^3\]H\]CGP-12177 (70 nM). Binding was performed at 4 °C for 6 h. The cells were diluted, collected on GF/C membranes at reduced pressure, and washed rapidly. The radioligand bound to the washed cell mass on the filter represents a direct assay of the cell surface complement of receptors. The amount of bound ligand was quantified by liquid scintillation spectrometry (18, 19).

RESULTS

N-Myristoylation of Gravin Is Not Required for Function—Human gravin possesses the N-terminal sequence N-
MGAGSSTEQR, a well known signal recognition sequence for N-myristoylation (5, 20), a co-translational modification affording weak protein-membrane interactions. Gravin (Mr ~250,000) was engineered either with the hemagglutinin tag (HA-gravin) at the N terminus (effectively precluding N-myristoylation) or as a fusion protein with the enhanced green fluorescent protein at the C terminus (gravin-GFP, Mr ~275,000), and stably expressed in human epidermoid carcinoma A431 cells (Fig. 1A). Confocal microscopy of cells shows HA-tagged gravin and gravin-GFP throughout the cell, except in the nucleus, and prominent at the cell membrane (Fig. 1B). Membrane-associated gravin is labeled with white arrows, whereas the cytosolic gravin is labeled with yellow arrowheads. The macroscopic pattern of localization for gravin was unaffected by the loss of the N-myristoylation (Fig. 1B).

To test the functional role of N-myristoylation itself on gravin function, we compared the functional capabilities of wild-type (WT) gravin to that of a gravin-GFP (G2A) mutant. To assess function, we first treated cells with isoproterenol (10 μM, 30 min; +ISO) to promote agonist-induced desensitization and internalization of β2AR (Fig. 1C). Cells made deficient of gravin (knockdown, KD) through treatment with antisense morpholinos fail to display recycling of agonist-stimulated internalized β2AR (gravin KD), as measured by radioligand binding experiments with the cell-impermeant β-adrenergic antagonist [3H]CGP-12177 that can only access cell surface β2AR. At 60-min postwashout of the isoproterenol (W60), knockdown of gravin attenuates the recycling of internalized β2AR. β2AR internalized in response to agonist treatment do not recycle in the gravin-KD cells (Fig. 1C). We compared the ability of expression of wild-type (myristoylated) gravin versus that of the G2A mutant of gravin (non-myristoylated) to rescue the recycling of internalized β2AR in cells in which endogenous gravin was suppressed. Expression of either the wild-type gravin or the G2A gravin mutant reconstituted the ability of the cells to rescue the recycling of the internalized β2AR. These observations demonstrate that N-myristoylation itself is not required for gravin function. Similarly, gravin tagged on the N terminus with the HA antigen has been shown to

FIGURE 1. Blockade of gravin N terminus and N-myristoylation does not alter cell membrane localization. A431 cells were transfected with expression vector harboring either gravin tagged at the N terminus with the HA antigen (HA-gravin) or a fusion protein of gravin fused C-terminally with enhanced green fluorescent protein (gravin-GFP), A, whole cell lysates were prepared and subjected to SDS-PAGE, transferred to nitrocellulose, and the blots probed with an antibody against gravin, against the HA antigen, or against the GFP moiety. B, A431 clones stably expressing the gravin-GFP and the HA-tagged gravin were analyzed by confocal microscopy in the unstimulated, basal state. The HA-tagged gravin was stained with anti-HA antibodies and a fluorescent secondary antibody. The results are typical of more than five separate experiments performed on as many separate clones. Gravin or gravin mutants associated with the cell membrane are labeled with white arrows, whereas those found in the cytoplasmic compartment are labeled with yellow arrowheads. C, A431 cells were untreated (Control) or treated with antisense morpholinos against gravin to knock down the expression of endogenous gravin (gravin KD). The antisense morpholino-treated cells were employed as such or transiently transfected with an expression vector harboring a gravin G2A mutant deficient (gravin-GFP (G2A)) or a full-length, wild-type gravin (WT gravin). Cells were desensitized with β-adrenergic agonist (isoproterenol 10 μM, + ISO) for 30 min and the amount of cell surface-associated β2AR measured either using the cell impermeant radiolabeled antagonist CGP as a ligand. Following 30 min of agonist treatment, half of the cells were washed free of the isoproterenol (Wash out) and incubated for a 60-min recovery phase of desensitization when the receptors recycle to the cell membrane. Cell surface-associated CGP binding was measured after the 60 min washout and recovery phase. The results, displayed as mean values ± S.E., are of at least three separate experiments performed with as many separate cultures of A431 cells.
function normally (3). Thus wild-type gravin, unlike Src and MARCKS (which use myristate and a single PCD), does not require the myristate for membrane localization and function, although we cannot rule out that N-myristoylation might facilitate some other subtle albeit important aspect of membrane localization for the AKAP. In view of these data, we adopted the use of the HA-tagged gravin which cannot be myristoylated in some of these studies.

Identification of Three MARCKS Effector Domain-like Regions in Gravin—We and others have identified three positively charged domains (PCDs) in gravin (4, 6) that are similar to the positively charged “effector domain” of MARCKS (21). These PCDs all contain both basic (blue: Lys, Arg) and hydrophobic (green; Phe, Met, Leu, Val, Cys, Trp) residues, but lack acidic residues: PCD1 (171GFKKVFKVFVFKF187); PCD2 (297KKFTQGWAGWRKKTSPFKPK317); and, PCD3 (510KVGSLKLLFTSTGLKKLSGKKQKGK536). These PCDs are found in the N terminus of gravin and in the mouse SSeCKS (4) (Fig. 2A). We hypothesize each of these regions can act in a manner similar to the effector domain of MARCKS protein, i.e. bind electrostatically to acidic lipids on the inner leaflet of the plasma membrane and have this binding reversed by Ca\(^{2+}\)/CaM (21). Analysis of the N-terminal domain of gravin using the programs PONDR (22) and FOLDINDEX (23) (as well as a simple coloring scheme: red, acidic; blue, basic; green, hydrophobic) suggests the entire region is “natively unfolded” (24).

**FIGURE 2.** Sequence location and CaM binding potential of three small positively charged domains (PCD1, PCD2, and PCD3) of human gravin. A, the sequence of human gravin was scanned for two motifs, the effector domain of the MARCKS protein and for CaM binding sites. Two other landmarks of gravin are displayed: the AKAP sites that constitute the β2-adrenergic receptor binding domain; and the binding site for the RII subunit of PKA. These data were obtained using ProSite software. B, full-length HA-tagged, C-terminal and N-terminal truncated gravin mutants were generated for analysis of their ability to bind to CaM immobilized to Sepharose-4B in the presence of Ca\(^{2+}\). **Upper panel**, HA-tagged gravin and mutant forms were expressed in A431 cells, whole cell lysates of the cells were subjected to SDS-PAGE, immunoblotted, and stained with anti-HA antibodies. Lower panel, fragments of gravin that were retained by immobilized CaM matrix, released from the matrix using buffer containing EDTA, subjected to SDS-PAGE, immunoblotted and stained with anti-HA antibodies. The data presented are representative of at least three separate determinations, each performed with separate cell lysates.
That is, it has a high density of negatively charged residues and a paucity of either basic or hydrophobic residues (except for the three PCDs). This N-terminal region of gravin is similar to the MARCKS protein, which is natively unfolded and is highly negatively charged except for the single basic/hydrophobic effector domain. The three PCD regions of gravin, and the comparable region of AKAP79 (31KASMLCFKRRKAAKALKPKAG52) (25), all contain both basic and hydrophobic residues, but lack acidic residues. Hence they would be predicted to bind electrostatically to the negatively charged inner leaflet of the plasma membrane and have the potential to bind Ca\(^{2+}\)/CaM with biologically significant affinity. Although others have speculated that CaM may bind to sequences common to gravin and AKAP79, the role of Ca\(^{2+}\)/CaM in regulation of AKAP-based signaling, localization, and function is not fully understood (4).

The working hypothesis is that each of the three PCDs helps anchor gravin to the membrane (6), and that each binds Ca\(^{2+}\)/CaM with significant affinity to reverse the membrane binding. PCDs help direct the proteins such as K-Ras4B to the plasma membrane rather than to internal membranes, as the former has a more negative electrostatic surface potential (26, 27).

We first examined HA-gravin and HA-gravin mutants with targeted deletions of the basic putative Ca\(^{2+}\)/CaM binding PCDs. The constructs were designed (Fig. 2B), expressed in A431 clones and tested to determine qualitatively if they were capable of binding Ca\(^{2+}\)/CaM (Fig. 2C, upper panel). Full-length gravin (1–1782), the N terminus (1–362) harboring PCD1 and PCD2, as well as the larger gravin (1–652) C-terminal truncated harboring PCD1, PCD2, and PCD3 all displayed binding by immobilized CaM-Sepharose 4B (CaM-matrix). The 554–938 region of gravin also displayed the capacity to bind CaM-matrix and release by exposure to molar excess of EDTA (Fig. 2C, lower panel). The C-terminal region of the AKAP (840–1783), in contrast, failed to bind to the immobilized CaM matrix (Fig. 2C). Similar experiments were performed with the three PCD peptides (minus flanking gravin sequences). Each peptide was successfully expressed in A431 cells and each showed qualitatively the same retention by the CaM matrix and release by exposure to molar excess of EDTA (data not shown). The affinity of these gravin fragments for Ca\(^{2+}\)/CaM cannot be readily deduced from these CaM matrix experiments. A more quantitative test of the hypothesis was conducted by studying directly the binding of synthetic PCD peptides of gravin to phospholipid vesicles.

We synthesized radiolabeled peptides corresponding to gravin PCD1, PCD2, and PCD3 and measured their membrane and Ca\(^{2+}\)/CaM binding affinities. The inner leaflet of a mammalian plasma membrane typically contains between 15–30% acidic lipid, mainly phosphatidylserine. Thus we measured the equilibrium binding to 5:1 and 2:1 phosphatidylcholine (PC)/phosphatidylserine (PS), large unilamellar vesicles 100 nm diameter, LUVs) using a centrifugation technique (8). The results of the membrane binding assay for PCD3 show that this peptide binds strongly to the PC/PS vesicles (Fig. 3A). The affinity increases with the mol fraction of negatively charged PS in the vesicles: it binds 50-fold more strongly to the 2:1 than to the 5:1 PC/PS vesicles. Similar measurements on vesicles containing 10 and 25% acidic lipid (not shown), when combined with the data in Fig. 3A, reveal the binding affinity increases exponentially with the fraction of acidic lipid in the membrane (binding energy increases linearly with fraction of PS), as expected theoretically for nonspecific electrostatic interactions (15) and observed experimentally for the MARCKS effector domain peptide (28) and other basic/hydrophobic peptides (21). PCD1 and PCD2 each bind with qualitatively similar affinities to 5:1 PC/PS vesicles as does PCD3 (Table 1). In other experiments (not shown), we observed that the affinity of PCD1 to bind PC vesicles that contain only 1% PIP2 was even higher than that for PC/PS vesicles that contain 17% PS (Table 1).
Ca\(^{2+}\)/CaM, but not apocalmodulin, binds to the PCD3 peptide with sufficient affinity to prevent its association with the membrane (Fig. 3B). In the absence of calmodulin, we chose conditions such that 90% of the peptide is bound to the vesicles or \(K \times [\text{lipid}] = 10\): note that 100 nM Ca\(^{2+}\)/CaM removes 50% of the peptide from the membrane, which implies that \(K_{\text{Ca/CaM}} = 10\) or that the \(K_d\) of the Ca\(^{2+}\)/CaM peptide complex is \(\sim 10\) nm. Specifically, the curve is drawn according to Equation 2 (see “Experimental Procedures”) with \(K_{\text{Ca/CaM}} = 10^8\) M\(^{-1}\). The affinity of all three of the PCD peptides for Ca\(^{2+}\)/CaM is very similar (Fig. 3, C and D). The Ca\(^{2+}\)/CaM affinities \((K_d \sim 10\) nM\) for all three peptides (see Table 1) are sufficiently high to be of biological significance, i.e. strong enough to displace the protein from the membrane even though most of the Ca\(^{2+}\)/CaM in cells may be bound rather than free in the cytosol (29, 30).

**Intracellular Calcium Regulates Membrane Localization of Gravin**—The ability of Ca\(^{2+}\)/CaM to neutralize the binding of gravin PCD1, PCD2, and PCD3 peptides to vesicles *in vitro* prompted study of the effects of changing intracellular concentrations of free Ca\(^{2+}\) on localization of the AKAP gravin. To increase intracellular Ca\(^{2+}\), cells expressing native (i.e. myristoylated) gravin-GFP were treated with the Ca\(^{2+}\) ionophore A23187 in the presence of normal extracellular Ca\(^{2+}\). In the absence of the Ca\(^{2+}\) ionophore, gravin-GFP is observed throughout the cytoplasm (yellow arrowheads) and in abundance at close proximity to the cell membrane (white arrows, Figs. 1B and 4A, *left panel*). Thirty minutes after ionophore/ Ca\(^{2+}\) treatment, cells assumed a more rounded morphology and gravin-GFP was found to redistribute uniformly throughout the cytoplasm (Fig. 4A, *center panel*). Thus, increasing intracellular concentration of Ca\(^{2+}\) decreases the ability of gravin to localize to the cell membrane. Complementary studies in cells preloaded with the Ca\(^{2+}\)-buffering agent BAPTA-AM ester demonstrated gravin localization prominently at the cell membrane (Fig. 4A, *right panel*).

A 1–362 C-terminally truncated gravin harboring PCD1 and PCD2 (but not PCD3) was fused with the cyan fluorescent protein CFP (1–362 gravin-CFP, Fig. 4B). As noted for gravin, the 1–362 gravin-CFP displays localization to the membrane and to the cytoplasm. Treating cells with Ca\(^{2+}\) ionophore provoked a sharp loss in the amount of cell membrane-associated 1–362 gravin-CFP. Buffering the intracellular Ca\(^{2+}\) with BAPTA, in contrast, increased the amount of 1–362 gravin-CFP associated with the cell membrane, mimicking the Ca\(^{2+}\)-dependent localization of the full-length AKAP to the membrane (compare *panels A* and *B*, Fig. 4). These results show that two PCDs are sufficient to essentially mimic the membrane binding properties of the wild-type gravin protein, in agreement with independent work (6).

If membrane binding of gravin is required for its interaction with receptor, decreasing the membrane binding by increasing the level of [Ca\(^{2+}\)]\(_i\), should inhibit the ability of

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**FIGURE 3. Interactions of synthetic peptides corresponding to gravin PCDs to phospholipid vesicles: analysis of binding and Ca\(^{2+}\)/calmodulin sensitivity.** A, binding of radiolabeled synthetic peptide of gravin PCD3 (corresponding to residues 510–536) to phospholipid vesicles (LUVs) of 2:1 (●) and 5:1 (●) PC/PS composition. The aqueous solutions contain 100 mM KCl, 1 mM MOPS, pH 7. The % peptide bound is determined from a centrifugation assay. The curves through the points illustrate the fit of Equation 1 to the data. The reciprocal of the lipid concentration that binds 50% of the peptide is the molar partition coefficient, \(K\). For the 5:1 PC/PS vesicles \(K = 10^4\) M\(^{-1}\), as indicated by the *dashed line*. For the 2:1 PC/PS vesicles, \(K = 5 \times 10^5\) M\(^{-1}\). A, Ca\(^{2+}\)/CaM (●), but not apocalmodulin (●), binds strongly to PCD3 and can reverse the binding to 2:1 PC/PS vesicles. The % membrane bound peptide is plotted as a function of the concentration of calmodulin in the presence (free Ca\(^{2+}\) = 20 nM (●)) or absence (○) of Ca\(^{2+}\). The solutions contain 100 mM KCl, 1 mM MOPS, pH 7, 100 mM EGTA = 120 mM CaCl\(_2\). The curve illustrates the prediction of Equation 2 with the association constant of the peptide with Ca\(^{2+}\)/CaM, \(K_{\text{Ca/CaM}} = 10^8\) M\(^{-1}\) (dissociation constant \(K_d = 10\) nM). C and D, Ca\(^{2+}\)/CaM reverses membrane binding of PCD1 (panel C) and PCD2 (D) peptides, corresponding to residues 171–187 and 297–317 of gravin, respectively. Binding to PC/PS (5:1) vesicles was measured in either the presence or absence of Ca\(^{2+}\). The % peptide bound is plotted as a function of the concentration of calmodulin in the presence (free Ca\(^{2+}\) = 20 nM (●)) or absence (○) of Ca\(^{2+}\). The solutions contain 100 mM KCl, 1 mM MOPS, pH 7, 100 mM EGTA = 120 mM CaCl\(_2\). The curves represent the best fit of Equation 2 to the data; the calculations take into account the competition between membrane and Ca\(^{2+}\)/CaM for the peptide. The association constants of the PCD1 and PCD2 peptides with Ca\(^{2+}\)/CaM deduced from these fits are \(K_{\text{Ca/CaM}} = 5 \times 10^7\) M\(^{-1}\) and 2 × 10\(^8\) M\(^{-1}\), respectively; apparent \(K_d = 20\) and 5 nM, respectively.
The functional role of AKAP's membrane association and of these small, positively charged PCDs was explored. Gravin acts as a "tool box" for a variety of docking proteins, including protein kinases and phosphoprotein phosphatases, that are necessary for normal biology of the β2AR (2). In cells expressing endogenous gravin, β2AR are internalized within 30 min of treatment with agonist (isoproterenol 10 μM, + ISO). Binding of the cell-impermeant [3H]CGP-12177 antagonist ligand again was used to quantify the amount of cell surface β2AR (Fig. 5A). After a washout of agonist and a recovery period, CGP binding in control (Control) cells returns to normal within 60 min, as the β2ARs are recycled back to the cell membrane. In cells made deficient of gravin by knockdown with antisense morpholinos (gravin-KD), β-adrenergic agonists stimulate desensitization/internalization of β2ARs normally (Fig. 5A), whereas resensitization/recycling of the receptor to the membrane is lost (Fig. 5A) (3, 31, 32).

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Confocal microscopy imaging of eGFP-tagged β2AR confirmed the data obtained with the [3H]CGP-12177 binding (Fig. 6A), i.e., loss of two PCDs virtually abolishes the ability of the gravin to function in receptor recycling. β2AR-eGFP located in the cell membrane is labeled with white arrows, whereas internalized β2AR-eGFP is labeled with yellow arrowheads. In gravin-deficient cells, expression of wild-type gravin, but not ΔPCD1,2-gravin, rescues the ability of these cells to recycle internalized receptors to the cell membrane. Deletion of a single PCD alone (i.e. ΔPCD3) from gravin, in contrast, did not significantly alter the localization or the function of gravin (data not shown). The simplest interpretation is that two positively charges clusters (i.e., PCD1, PCD2, or PCD3) are required in tandem for membrane association and function of the HA-tagged gravin.

Gravin Requires Two PCDs to Function in Recycling of β2-Adrenergic Receptors—The functional role of AKAP's membrane association and of these small, positively charged PCDs was explored. Gravin acts as a “tool box” for a variety of docking proteins, including protein kinases and phosphoprotein phosphatases, that are necessary for normal biology of the β2AR (2). In cells expressing endogenous gravin, β2AR are internalized within 30 min of treatment with agonist (isoproterenol 10 μM, + ISO). Binding of the cell-impermeant [3H]CGP-12177 antagonist ligand again was used to quantify the amount of cell surface β2AR (Fig. 5A). After a washout of agonist and a recovery period, CGP binding in control (Control) cells returns to normal within 60 min, as the β2ARs are recycled back to the cell membrane. In cells made deficient of gravin by knockdown with antisense morpholinos (gravin-KD), β-adrenergic agonists stimulate desensitization/internalization of β2ARs normally (Fig. 5A), whereas resensitization/recycling of the receptor to the membrane is lost (Fig. 5A) (3, 31, 32).

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Gravin association with the cell membrane and its role in receptor resensitization/recycling are regulated by intracellular concentration of Ca\(^{2+}\). A and B, the full-length gravin (gravin-GFP) and the gravin fragment harboring PCD1 and PCD2, and the PCD3 (1–362-gravin-CFP) N-terminal region of gravin were created as autofluorescent fusion proteins, expressed in A431 cells and examined by confocal microscopy. Cells expressing either gravin-GFP (A) or 1–362-CFP gravin (B) were studied either without treatment (Basal), or after a 30-min treatment with the Ca\(^{2+}\) ionophore A23187 in the presence of normal extracellular Ca\(^{2+}\) to raise intracellular Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\)) (A23187), or after a 6-h prior treatment with the Ca\(^{2+}\)-buffering agent BAPTA-AM ester to reduce intracellular free Ca\(^{2+}\) concentrations (BAPTA). Gravin and mutant gravin associated with the cell membrane is decorated with white arrows. Gravin or mutant gravin localized to the cytoplasm is decorated with yellow arrowheads. The results are representative of at least five separate experiments.

C and D, cells were challenged with β-adrenergic agonist (isoproterenol, 10 μM) to provoke agonist-induced desensitization and internalization. The agonist was washed out and the cells were either untreated (Control) or treated to raise intracellular [Ca\(^{2+}\)]\(_i\) using the Ca\(^{2+}\) ionophore A23187 in the presence of normal extracellular [Ca\(^{2+}\)] or using the α\(_1\)-adrenergic agonist phenylephrine (100 μM). The recovery from agonist-induced desensitization, termed “resensitization,” was measured in cells at 30 (W30), 45 (W45), or 60 (W60) min following the washout. Isoproterenol-stimulated cyclic AMP accumulation was measured in these cells and the amount of cyclic AMP accumulation obtained with full recovery set as 100% (C). Parallel experiments were performed in which the amount of β\(_2\)AR lost from the cell surface, termed “internalized”, in response to agonist-induced treatment was set as 100% (D). The results, displayed as mean values ± S.E., are of at least three separate experiments performed with as many separate cultures of A431 cells.
for pull-downs with HA-gravin. Although direct contact sites of gravin for the receptor map to the RBD located C-terminal to PCD1 and PCD2 (3), the presence of PCD1,2 is essential for full scaffold-receptor interactions (Fig. 6B). Thus, for the non-myris-toylated gravin, interaction with the plasma membrane-localized GPCR requires at least two of the three PCDs.
DISCUSSION

In the current work we explored how gravin PCDs bind lipid vesicles, whether or not Ca\(^{2+}\)/calmodulin alters gravin binding to membranes, and how membrane association impacts gravin-mediated \(\beta_2\)ARs resensitization and recycling after agonist-induced desensitization/internalization. Binding of gravin to the receptor under unstimulated conditions would appear too weak to occur unless gravin is bound to the inner leaflet of the cell membrane (Fig. 7). When gravin is bound to the cell membrane, the receptor-gravin interaction would benefit by a \(\sim 1000\)-fold increase in the local concentration of the scaffold with the membrane-embedded \(\beta_2\)AR (Fig. 7A). Agonist binding to the \(\beta_2\)AR activates the stimulatory adenyl cyclase pathway and the PKA tethered to gravin (3). Activated PKA phosphorylates both the scaffold and receptor, dramatically enhancing their protein-protein interactions (Fig. 7B). Although not required itself for gravin function, myristate may facilitate further the interactions between the membrane and two PCDs required for binding. We cannot rule out the potential importance of the myristate in either membrane anchoring or lateral distribution, although the \(\beta\)- and \(\gamma\)-isoforms of gravin, lacking N-terminal myristoylation, appear to localize as does gravin (6).
Our localization studies with gravin-GFP, compare well with those of gravin-β-GFP (6). We measured quantitatively the binding affinities of peptides corresponding to each PCD in gravin to phospholipid vesicles and Ca^{2+}/CaM. (As with the basic region of MARCKS, the PCDs in gravin probably bind to membranes with only about half the energy of the PCD peptides because flanking acidic residues in the protein reduce electrostatic interactions.) By functional studies we were able to show that the presence of two PCDs is essential to the ability of the scaffold in the RBD (3). The increase in intracellular Ca^{2+} concentrations neutralizes the membrane association of these three domains enabling the binding of CaM, repelling the scaffold from the cell membrane as the phosphorylated receptor and RBD of gravin enhance the interaction of the scaffold harboring PKA, PKC, and the phosphatase PP2B. Following the transient rise and fall of intracellular Ca^{2+} levels, the small, positively charged domains drive membrane association of the scaffold again and enable the resensitization (dephosphorylation) and recycling of the receptor as well as dephosphorylation of the AKAP.

Elevating intracellular Ca^{2+} decreases the association of gravin with the cell membrane and blocks the ability of gravin to resensitize and recycle the β_{2}AR. Responses integrating signals provoked by changes in intracellular concentrations of cyclic nucleotides and free Ca^{2+} often are triggered in vivo by neurotransmitters such as norepinephrine, operating through both α_{1}- and β_{2}-adrenergic receptors. Catecholamines epinephrine and norepinephrine stimulate both β_{2}-adrenergic receptors (elevating cyclic AMP levels and activating PKA) and α_{1}-adrenergic receptors (increasing intracellular Ca^{2+} concentrations transiently). Gravin is shown to be a scaffold integrating signaling via cyclic nucleotides and intracellular Ca^{2+} concentrations, ultimately favoring trafficking of the scaffold away from the cell membrane during a Ca^{2+} transient (Fig. 7B), modulating a process that appears essential for receptor recycling (3, 32). The fall of intracellular [Ca^{2+}]_{i} (after the Ca^{2+} transient) would result in a reduction in the neutralization of PCDs by Ca^{2+}/CaM (Fig. 7C), restoring the movement of gravin from the cytoplasmic compartment to the cell membrane. A recent model for NMDA receptor regulation of MAGUK-AKAP79/150-based signaling likewise suggests the operation of a Ca^{2+}-sensitive association of the scaffold with the cell membrane (33, 34). These two studies highlight the central role of short, positively charged domains and Ca^{2+} in regulating the localization of AKAPs, such as gravin and AKAP79/150, at the cell membrane and provide further information on the broader topic of how multivalent scaffolds like gravin and AKAP79/150 integrate signals emanating from different signaling cascades.

Earlier mutagenic studies of the non-myristoylated β- and γ-isofoms of AKAP12 suggested that two of the three PCDs in the gravin-γ N-terminal region are necessary for plasma membrane association, as determined by confocal microscopy (6). Deleting any one cluster decreased membrane association modestly, deleting any two clusters decreased membrane association further (6). Substitution of acidic residues for Ser and Thr residues in these basic regions of gravin-γ, mimicking PKC phosphorylation, also reduced apparent association with the membrane (6). The current work extends these earlier findings (6) by an investigation of the membrane binding and role of PCDs in the function of this AKAP scaffold.

For MARCKS, either phosphorylation by PKC or Ca^{2+}/CaM binding to the effector domain stimulates translocation of MARCKS from the plasma membrane to the cytosol (12, 21). The N-terminal region of gravin and the entire MARCKS protein dis-
AKAP Gravin, Ca\(^{2+}\), and Scaffold Function

play a highly acidic, apparently unfolded region with one (MARCKS protein) or three (gravin) basic/hydrophobic domains that target the protein to the plasma membrane through electrostatic interactions with acidic lipids. We show (Fig. 3) that Ca\(^{2+}\)/CaM can bind with sufficiently high affinity (K_d \approx 10 \text{ nm}) to each of the PCDs to release these peptide sequences from the membrane. Similarly, inserting negatively charged residues into PCDs to mimic PKC phosphorylation decrease interactions between PCDs and the cell membrane, producing translocation of AKAP from the membrane to the cytosol (6). In summary, our measurements and the confocal microscopy results support our hypothesis that local concentrations of Ca\(^{2+}\) (and thus Ca\(^{2+}\)/CaM) can regulate association of the AKAP gravin (via these basic regions) with the inner leaflet of the plasma membrane.

The first 150 residues of AKAP79 include three clusters of basic residues proposed to function as a "polybasic membrane targeting domain" (11). In AKAP79, the sequence \text{31KASMLCFRRK-KAAKALPKKAG}^\text{52}, also has been directly implicated as a CaM binding domain (25). Previous studies of the regulation of interactions of AKAP79 with protein kinase C, phosphatidylinositol 4,5-bisphosphate, F-actin, and cadherins by protein phosphorylation and by Ca\(^{2+}\)/CaM support the tenet that the N-terminal, three PCDs in the membrane targeting domain of this AKAP are negatively regulated by Ca\(^{2+}\)/CaM binding (10, 25, 33, 35). Gravin uses three types of interactions to anchor itself to the plasma membrane: (i) the comparatively weak hydrophobic insertion of the N-terminal myristate for some isoforms, (ii) electrostatic interaction of the three PCDs with acidic lipids, and (iii) protein–protein interactions with the GPCR \(\beta_2\)AR. The protein–protein interaction of scaffold with the receptor (i.e. mediated by RBD binding) is dynamic and regulated by protein phosphorylation/dephosphorylation (3). The PCD–membrane interaction is dynamic and regulated by Ca\(^{2+}\)/CaM binding to the PCDs. The PCDs in gravin function to increase the local concentration of the AKAP at the cell membrane and thereby enhancing the protein–protein interactions between the scaffold and this GPCR (36). Our observations demonstrate that gravin functions as a scaffold integrating signals from two major pathways, \(\beta_2\)AR/G_{\alpha_\text{i}}/AC/cyclicAMP and \(\alpha_\text{1}/\alpha_\text{3}/G_{\text{q}}/PLC\beta/Ca^{2+}\).

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