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Gravin Regulates Crosstalk Between Calcium And PkA Dependent Signaling Pathways In Cultured Cells

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GRAVIN REGULATES CROSSTALK BETWEEN CALCIUM AND PKA
DEPENDENT SIGNALING PATHWAYS IN CULTURED CELLS

by

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This dissertation, submitted by Micah B. Schott in partial fulfillment of the requirements for the Degree of Doctor of Philosophy from the University of North Dakota, has been read by the Faculty Advisory Committee under whom the work has been done and is hereby approved.

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Title: Gravin Regulates Crosstalk Between Calcium and PKA Dependent
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Summer and winter and springtime and harvest,
sun, moon, and stars in their courses above;
join with all nature in manifold witness
to Thy great faithfulness, mercy and love.

– Thomas Chisholm, 1923

ABSTRACT

A-Kinase Anchoring Proteins (AKAPs) comprise a family of roughly 70 scaffolds that anchor PKA and other enzymes to a variety of subcellular compartments. Although characterized as "anchoring proteins", some AKAPs are not spatially and temporally static but can undergo dynamic subcellular trafficking, thus repositioning anchored enzyme complexes within the cell. Gravin (also called SSeCKS or AKAP12) anchors PKA and other enzymes to the plasma membrane but redistributes to the cytosol upon intracellular calcium ($[Ca^{2+}]_i$) elevation. However, the impact of gravin redistribution on PKA-dependent signaling pathways is poorly understood. We hypothesize that through Ca^{2+}-mediated redistribution, gravin facilitates cross-talk between Ca^{2+}-dependent and PKA-dependent pathways.

First, we tested this by characterizing the impact of $[Ca^{2+}]_i$ elevation on the distribution of gravin and PKA. In cells expressing gravin-EGFP, $[Ca^{2+}]_i$ elevation with ionomycin or thapsigargin caused gravin redistribution from the cell periphery to the cytosol in as little as 60 seconds. ATP treatment also triggered gravin redistribution through receptor-mediated pathways involving both $[Ca^{2+}]_i$ and PKC. Gravin redistribution in response to ionomycin, thapsigargin, and ATP each triggered the gravin-dependent loss of PKA localization at the cell periphery. In addition, we also found that a fourth putative calmodulin binding domain, which we call CB4 (a.a. 669-693), is essential for localization of gravin to the cell periphery. Either deletion of the CB4

domain or mutation of a calmodulin-binding consensus sequence within the CB4 domain disrupted the membrane localization of gravin.

Next, we measured the impact of exogenous gravin-V5/His expression on compartmentalized PKA activity using the PKA FRET biosensor AKAR3. Expression of gravin-V5/His in AN3 CA cells, which lack endogenous gravin, caused an increase in forskolin-stimulated PKA activity at the plasma membrane when compared to control cells lacking gravin. Under these conditions, gravin also decreased PKA activity in the cytosol. Gravin's impact on subcellular PKA activity required both interaction with PKA and localization at the cell periphery. Pre-treatment with the $[Ca^{2+}]_i$ elevating agent thapsigargin caused gravin redistribution and inhibited gravin-mediated elevation of PKA activity the plasma membrane. These results support the hypothesis that gravin mediates crosstalk between Ca^{2+}-dependent and PKA-dependent signaling pathways.

CHAPTER I

INTRODUCTION

A-Kinase Anchoring Proteins

The most fascinating characteristic of the cell is its ability to transduce

extracellular inputs into very specific outputs through complicated networks of signaling

proteins, molecules and ions. Intracellular signal transduction is fundamental to cellular

homeostasis and relies on precise physical interactions between proteins within an input-

directed signaling cascade. Many signaling proteins have a range of downstream targets

but no intrinsic ability to specify which of these targets are appropriate for a given input.

How do cells facilitate the specificity of signaling proteins? In part, the answer to this

question lies in the spatial positioning of signaling proteins in close proximity to specific

downstream effectors (Good, Zalatan, & Lim, 2011). A prototypical example of this is

found in the A-Kinase Anchoring Proteins, or AKAPs, a specialized family of over 70

scaffolds which play an important role organizing intracellular signaling networks

(Welch, Jones, & Scott, 2010).

AKAPs share two common structural characteristics: the ability to bind cAMP-

dependent Protein Kinase A (PKA) and the ability to localize within a specific

subcellular compartment. This localization "anchors" PKA in proximity to downstream

substrates for phosphorylation, thereby providing spatial and temporal specificity to

PKA-dependent signaling. PKA is a holoenzyme consisting of two catalytic (C) subunits

that are bound to two regulatory (R) subunits. AKAPs bind to the R subunit through a

conserved amphipathic helical domain, and upon activation by 3'-5'-cyclic adenosine

monophosphate (cAMP), the R subunit releases the C subunit to phosphorylate substrate

proteins nearby. The interaction of AKAPs with PKA is critical to many PKA-dependent

processes. In fact, studies now show that disrupting AKAP-PKA interaction can have the

same effect as inhibiting PKA directly (Nijholt et al., 2008; Schillace et al., 2009; Y.

Wang, Chen, Chen, & Xu, 2006). Since PKA is predicted in *Homo sapiens* to

phosphorylate over 900 sites on 240 different proteins (Gao, Jin, Ren, Yao, & Xue,

2008), the apparent control of AKAPs over PKA signaling gives AKAPs a powerful role

in regulating many cellular functions.

Equally important to AKAP function is the subcellular localization motif, which

is varied among AKAPs and targets each to a specific compartment. These include the

plasma membrane, mitochondria, Golgi apparatus, nuclear envelope, and cytosolic

vesicles, or to other localized proteins such as cytoskeletal proteins, centrosomal proteins,

or membrane receptors (Wong & Scott, 2004). AKAPs target PKA to these subcellular

compartments and facilitate the PKA-dependent phosphorylation of substrates in close

spatial proximity within these compartments. In addition, many AKAPs bind not only

PKA, but a host of other kinases, phosphatases, and regulatory enzymes that may act

independently or within an anchored multiprotein signaling complex in which substrates

and downstream effectors are bound to the a single AKAP. One example of an anchored

multiprotein signaling complex is found in AKAPs that bind both PKA and

phosphodiesterase type 4D (PDE4D), a PKA substrate that hydrolyzes cAMP to

adenosine monophosphate. Upon elevation of [cAMP], activated PKA phosphorylates

PDE4D to enhance PDE4D activity. As a result, PDE4D activity causes the rapid

decrease in [cAMP], forming a negative feedback loop for [cAMP] elevation (Dodge et al., 2001).

Given the important physiological relevance of PKA signaling, the apparent control of PKA signaling by AKAPs, and the multitude of other enzymes bound to AKAPs, we expect the study of AKAPs will yield a robust understanding of cellular signaling in many cell and tissue contexts. The subject of this dissertation is the AKAP gravin, which is implicated to have a broad range of physiological roles. By examining the molecular signaling events in which gravin participates, my hope is to gain mechanistic insights into gravin's role in health and disease.

Gravin

Gravin, a 300kD AKAP, was discovered in the early 1990s while screening an endothelial expression library with serum from a patient with myasthenia gravis (Gordon et al., 1992). Subsequently, gravin expression has been detected in a variety of cells and tissues and has been implicated to play a role in cancer, vascular biology, cardiac function, learning and memory, inflammation, and tissue injury. However, the mechanism(s) behind these diverse roles are poorly understood, and this lack of knowledge is a serious barrier to potential therapeutic applications. Three isoforms of gravin (α, β, and γ) seem to vary in subcellular localization, but the canonical α-isoform is targeted to the plasma membrane where it is thought to facilitate not only PKA-dependent signaling, but signaling involving additional binding partners such as other kinases, regulatory enzymes, and β2-adrenregic receptor. Although gravin localizes PKA and other enzymes to the plasma membrane, it is known to undergo redistribution away from the plasma membrane in response to either elevation of intracellular calcium

concentration ($[Ca^{2+}]_i$) or upon activation of protein kinase C (PKC). Because AKAPs function in the spatial and temporal positioning of signaling enzymes, the redistribution of gravin may have a profound impact on how enzymes that bind to gravin, such as PKA, interact with specific downstream effectors. By changing its distribution in subcellular space, we postulate that gravin facilitates a crosstalk mechanism in which Ca^{2+} elevation and/or PKC activation can alter interaction of enzymes bound to gravin with their respective downstream targets.

Orthologues, Isoforms, and Nomenclature

Within the human genome, gravin is located on the q24-25.2 locus of chromosome 6 and contains six exons that are differentially spliced into three distinct isoforms, each with its own promoter (Streb, Kitchen, Gelman, & Miano, 2004; Streb & Miano, 2005). All three isoforms share exons 5 and 6, which makes up ~95% of the amino acid sequence. The canonical isoform, gravin-α, is a 1782 amino acid protein product with its N-terminal sequence derived from exons 1 and 2. These exons comprise an 88 amino acid region unique to gravin-α which contains an N-myristoylation sequence and a Src binding (PXXP) domain. The -β isoform (1682 amino acids) and the –γ isoform (1677 amino acids) contain N-termini from exons 3 and 4, respectively. Gravin-β and -γ lack these 88 amino acids, but still retain three polybasic domains (PB1-3) important for localization at the plasma membrane.

Gravin is expressed in many species including humans (Gordon et al., 1992), baboons (Grove, Bowditch, Gordon, del Zoppo, & Ginsberg, 1994), rodents (Frankfort & Gelman, 1995), zebrafish (Weiser, Pyati, & Kimelman, 2007) and Xenopus (Klingbeil, Frazzetto, & Bouwmeester, 2001). Human gravin was discovered in an endothelial cell

expression library screened with serum from a patient with myasthenia gravis, and was named gravin accordingly. (Gordon et al., 1992). Gravin was later called AKAP250 based on sequencing and biochemical data which revealed it to be an A-Kinase Anchoring Protein that aligned with a 250 kD marker on polyacrylamide gel (Nauert, Klauck, Langeberg, & Scott, 1997). The gravin gene was named AKAP12 by the Hugo Gene Nomenclature Committee (HGNC) and has also been used as an identifier of the protein product (Xia, Unger, Miller, Nelson, & Gelman, 2001). Gelman and colleagues discovered rodent gravin as a target of PKC phosphorylation which was suppressed in Src-transformed rat fibroblasts (Lin, Tombler, Nelson, Ross, & Gelman, 1996a). On this basis, it was named SSeCKS, or Src-Suppressed C-Kinase Substrate. The orthologous relationship between SSeCKS and gravin was not realized initially, but they in fact share 83% sequence identity within the first 1000 residues and <20% over the remaining C-terminus. Upon this realization, some authors sought to adapt the nomenclature using awkward names like "SSeCKS/gravin/AKAP12", but in recent years there is growing consensus for use of the name "gravin" across species. For example, knockdown mice and zebrafish studies have used the word gravin to name the gene target, which is especially helpful if multiple species are under investigation within a single study (for examples of this, see D. Canton et al., 2012; Guillory et al., 2013; Havekes et al., 2012; Isoldi, Provencio, & Castrucci, 2010; Weiser et al., 2007). Therefore, for this dissertation I will use the word "gravin" as the overlying nomenclature when referring to previous studies and indicate the species as needed. If I give no species specification, my use of the name "gravin" will be in reference to the alpha isoform that is expressed in humans.

Gravin expression is specific to certain cells and tissues, and this distribution seems to be consistent across various studies. In general, high expression of gravin is seen in nervous tissue, connective tissue, heart, smooth muscle, and gonads, while low or restricted expression is found in endothelium and other epithelia. Gravin is also differentially expressed during development. The following section will briefly outline each of these areas.

Nervous Tissue

Regions of the cerebral and cerebellar cortices show distinct cellular expression of gravin, with some reports of Purkinje cell expression and high expression in cells the molecular and granular layer of the cerebellum (Gelman, Tombler, & Vargas, 2000; Grove et al., 1994; Siegel, Grove, & Carr, 2002). In the hippocampus, gravin is expressed in the dentate gyrus as well as CA1 and CA3 regions (Havekes et al., 2012). Astrocytes also highly express gravin in an oxygen-sensitive manner (Lee et al., 2003). Siegel et al. (2002) additionally showed gravin expression in smaller-sized cell bodies of dorsal root ganglia and dorsal horn axons throughout the spinal cord, possibly indicating a role for gravin in pain sensation.

Heart and Vasculature

Gravin expression is found in cardiomyocytes but not in cardiac endothelium (Guillory et al., 2013). In general, endothelial cells show a restricted expression pattern of gravin *in vivo*. Nearly all endothelial cells show no gravin expression, with the exception of hepatic sinusoids and intestinal lacteals (Gelman et al., 2000; Grove et al., 1994; Rung-Ruangkijkrai, Fujikura, Kitamura, Saito, & Iwanaga, 2004). Gravin is expressed in

microvascular endothelial cells in culture (Weissmuller et al., 2014). In addition, gravin is expressed in human umbilical vein endothelial cells (HUVECs) in a cell density-dependent manner (Roy, Schott, and Grove, *in preparation*), which suggests that gravin may be important in vascular homeostasis. Smooth muscle cells also express gravin in various locations throughout the body, one of which is in the vasculature (Coats et al., 2000; Gelman et al., 2000; Grove et al., 1994; Horvat et al., 2012).

Epithelium

Like endothelium, most other epithelial cells do not express gravin with a few notable exceptions. Gelman et al. (2000) reported gravin expression in mouse jejunal epithelium, columnar epithelium of lung bronchioles, and certain portions of renal epithelium. Interestingly, the parietal layer of Bowman's capsule in the renal glomerulus has exceptionally high levels of gravin expression (Gelman et al., 2000; Grove et al., 1994).

Connective Tissue

Several reports show high gravin labeling in connective tissues both in adulthood and during development. Organ capsules have consistently high expression levels, as does the mesentery and lamina propria of the gut. The principle cell showing high expression in these tissues is the fibroblast, but gravin expression is also reported in osteoclasts and chondrocytes (Gelman et al., 2000; Grove et al., 1994).

Gonads

High levels of gravin are found in both the testes and the ovaries. The testis is the only reported location for gravin-γ, but gravin-α is also expressed in the testis (Streb et al., 2004). Another report shows that Sertoli cells express gravin, as well as spermatids

(Erlichman, Gutierrez-Juarez, Zucker, Mei, & Orr, 1999). Interestingly, delayed fertility was reported in one particular line of gravin knockdown mice (Akakura, Huang, Nelson, Foster, & Gelman, 2008) and may indicate the possibility that gravin is important for germ cell maturation. In the female mouse, gravin is also highly expressed in the ovaries (Lin, Nelson, & Gelman, 2000).

Miscellaneous

Within the kidney, in addition to the various epithelial cells discussed previously, high levels of gravin are found in mesangial cells of the glomerulus (Gelman et al., 2000; Grove et al., 1994; Nelson, Moissoglu, Vargas Jr, Klotman, & Gelman, 1999). Not far away from this location, cells of the adrenal medulla also express high levels of gravin (Grove et al., 1994). In the liver, gravin expression is reported not only in hepatic sinusoid endothelium, but also in hepatic stellate cells (Gelman et al., 2000; Grove et al., 1994; Jiang et al., 2008; T. You et al., 2013)

Expression during Development

During mouse development, gravin is highly expressed in mesenchyme and around neural precursors like the neural tube, neural crest, and in neural crest-derived spinal primordia. Gravin is also expressed in dorsal/ventral surfaces of limb buds and in other organs as development progresses (Gelman et al., 2000). In *Xenopus laevis*, Klingbeil et al. (2001) show that gravin is differentially expressed during development. Expression of *Xenopus* gravin begins stage 10 and continues throughout development with peak expression seen at stages 11-12. During gastrulation, gravin is distributed in posterior mesodermal cells on the dorsal lip of the blastopore (the Spemann organizer). As gastrulation progresses, this labeling pattern progressively distributes downward

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around the blastopore circumference. During *Xenopus* neurulation, gravin is expressed in the forebrain and on two bilateral neuroectodermal stripes at the midbrain/hindbrain boundary. Gravin is also found in the notochord, spinal cord, and in mandibular neural crest cells. At later stages of development, *Xenopus* embryos show predominant labeling in the heart and notochord (Klingbeil et al., 2001). The role of gravin in *Xenopus* development is completely unknown, but an interesting study by Weiser et al. (2007) suggests a critical role for gravin during gastrulation in zebrafish (*Danio rerio*). In this study, antisense morpholino knockdown of gravin dramatically reduced anterior-posterior axis extension by blocking the extension of paraxial mesodermal cells lateral to the notochord. This resulted in defective formation of the heart, pancreas, and liver.

Role of Gravin in Health and Disease

Many studies suggest that gravin plays a broad physiological role in health and disease. The majority of these studies are expression-based, and the lack of mechanistic insight provides a serious barrier to therapeutic application. Nonetheless, the apparent role of gravin in health and disease is quite impressive, not only in terms of the broadness of its impact, but also the importance that these physiological processes represent in human health. The following section will highlight what is known about gravin's role in health and disease states.

Regulation of Gravin Expression in Cancer

Oncogenic kinases v-Src and v-Jun induce tumorigenic phenotypes marked by mitogenic dysregulation and cytoskeletal remodeling in rodent fibroblasts. Early on, Gelman and colleagues discovered that Src- and Jun-induced transformation of these cells was accompanied by suppressed gravin expression (Frankfort & Gelman, 1995; Lin

et al., 1996a; Nelson & Gelman, 1997). Importantly, the re-expression of exogenous gravin in these transformed rat fibroblasts inhibited mitogenic dysregulation by reducing soft-agar growth and dramatically reducing proliferation rate. (Cohen, Waha, Gelman, & Vogt, 2001; Lin & Gelman, 1997). Subsequently, downregulation of gravin in cancer tissues has been widely reported. For example, microarray studies show suppressed gravin expression in cancerous lung (Tessema et al., 2008), thyroid (Wasenius et al., 2003), colon (Yildirim et al., 2013), and liver (Goeppert et al., 2010). Other studies show that gravin is suppressed in cancers derived from stomach (M. C. Choi et al., 2004), pancreas (Cao et al., 2004; Mardin et al., 2010), breast (Vaidya & Welch, 2007), skin (Bonazzi, Irwin, & Hayward, 2009), blood (Mostafa, Yahia, Abd El Messih, El-Sisy, & El Ghannam, 2013; Yildirim et al., 2007), and grade IV astrocytoma (Goeppert et al., 2013). These studies further reveal that the gravin is often silenced by epigenetic mechanisms in many types of cancer, and re-expression can be achieved by treatment with the demethylating agent 5'-Aza-dC or with the histone deacetylase inhibitor TSA.

Gravin's gene locus on chromosome six is considered to be a major hotspot for deletion in cancers, particularly advanced prostate cancer. Indeed, gravin is readily detected in secretory epithelium and the surrounding mesenchyme of normal prostate tissue, but is silenced in prostate cancer biopsies and a variety of prostate cancer cell lines (Xia et al., 2001). Subsequently, much information has been gained from a model system which uses the forced re-expression of exogenous gravin in MatLyLu prostate cancer cell lines, which are gravin-deficient. Forced gravin expression in these cells inhibits proliferation, soft agar growth, Boyden chamber chemotaxis, and matrigel invasion (Su, Bu, Engelberg, & Gelman, 2010; Xia et al., 2001). Injection of these cells into nude mice

further revealed that gravin re-expression inhibits tumorigenesis, metastasis, and angiogenesis in subcutaneous tumors through the downregulation of VEGF expression (Su, Zheng, Vaughan, Bu, & Gelman, 2006). Moreover, prostate tissue from gravin knockout mice showed hyperplasia in the anterior and ventral lobes which was concurrent with increased expression of markers for proliferation and apoptosis (Akakura et al., 2008). Mechanistic insights from these expression studies strongly suggest that gravin interacts with PKC, MEK, ERK, MMP-2, and E-cadherin pathways in prostate cancer. However, the precise nature of gravin's interaction with these signaling mediators in cancer progression is poorly understood.

Regulation of β2-adrenergic Receptor Sensitivity

Gravin's influence over β2-adrenergic receptor (β2AR) signaling has been studied extensively in the context of receptor resensitization (Reviewed by Malbon, Tao, & Wang, 2004). As a result, *in vivo* studies have now investigated this interplay in the hippocampus and the heart. In hippocampal brain slices of gravin-knock down mice, Havekes et al. (2012) demonstrated that β2AR mediated synaptic plasticity is altered at CA1 synapses. A variety of behavioral tests revealed that gravin knock-down mice displayed impairments in fear-conditioned long-term memory formation, and this corresponded with a reduction in phosphorylation of β2AR and ERK1/2 (Havekes et al., 2012). This study correlates well with the notion that gravin regulates β2AR sensitivity and sets the stage for future studies to investigate the intracellular dynamics between these two proteins in hippocampal learning and memory.

In the heart, Guillory et al. (2013) used echocardiography in a different mouse model to show that gravin knockdown enhances cardiac contractility under both basal

conditions and when treated with isoproterenol, a β2AR agonist. Cultured

cardiomyocytes from these mice had significantly altered calcium dynamics as well,

implying that gravin may regulate L-type calcium channels or sarcoplasmic reticulum

ryanodine receptors, both of which are phosphorylated by PKA. Interestingly, gravin

knockdown caused a dramatic increase in phosphodiesterase activity in response to

isoproterenol treatment, despite the fact that cellular cAMP levels and PDE4D3/5

expression were unchanged (Guillory et al., 2013). Although this study raises many

unanswered questions regarding the role of gravin in cardiac physiology, it is clearly

linked at least in part to β2AR signaling and will be an area of great interest in future

research.

Regulation of Vascular Endothelial Function

Grove and Bruchey (2001) reported that gravin binds both PKA and PKC in

human umbilical vein endothelial cells (HUVECs) and is distributed at the cell periphery

independent of cytoskeletal proteins (Grove & Bruchey, 2001). Data from our lab have

further revealed that subconfluent HUVECs readily express gravin, but confluent

monolayers do not. In fact, scratch wounded confluent HUVEC monolayers showed

elevated gravin expression at the wound edge, and gravin knockdown under these

conditions slowed the rate of wound closure (Roy, Schott, and Grove; *in preparation*).

This would imply that gravin enhances cellular migration, but the role of gravin in

endothelial cell migration may be more complicated. For example, Turtoi et al (2010)

suggested that HDAC7 knockdown in HUVECs leads to gravin overexpression and the

inhibition of cellular migration (Turtoi et al., 2012). It is clear that gravin plays a role in

cell migration, but future studies will be required to understand these differential results.

In addition to regulating endothelial cell migration, gravin is also shown to regulate endothelial barrier permeability. In human microvascular endothelial cells (HMEC), gravin inhibited the formation of endothelial tubules, and in response to GPCR agonists, gravin supported endothelial barrier strength (Weissmuller et al., 2014). In zebrafish embryos, gravin was also linked to the enhancement of endothelial cell-cell contacts (Kwon et al., 2012). Conversely, You et al (2010) showed that gravin may promote endothelial permeability in response to inflammatory cytokines. (Q. H. You, Sun, Wang, Chen, & Luo, 2010).

Gravin may also regulate endothelial cell function through supporting cells that associate with the vasculature. For example, gravin regulates vascular endothelial function indirectly through gravin-expressing astrocytes at the blood-brain barrier. A study by Lee et al. (2003) showed that gravin expression in astrocytes causes VEGF downregulation, and that conditioned medium from gravin-overexpressing astrocytes suppressed endothelial cell angiogenesis. In addition, conditioned medium from gravin-overexpressing astrocytes also stimulated the increased expression of tight junction proteins in endothelial cells, supporting cell-cell contact and resisting endothelial permeability (Lee et al., 2003). These results were confirmed in a similar study using gravin-overexpressing astrocytes (Y. K. Choi & Kim, 2008).

Regulation of Gravin Expression by Inflammatory Mediators

Many inflammatory mediators have been shown to increase gravin expression, but the physiological impact of this is poorly understood. Lipopolysaccharide (LPS) induces gravin upregulation in a variety of adherent cells and tissue models (Cheng et al., 2007; Kitamura et al., 2002; M. Yan et al., 2007; M. Yan et al., 2014). Gravin

upregulation seems to support LPS-mediated production of TNF-α, nitric oxide, iNOS, and the phosphorylation of ERK, p38, and JNK. (X. Li et al., 2010; Shao et al., 2011; Sun, Cheng, Liu, Shen, et al., 2007; P. Wang et al., 2010). Gravin was also shown to be upregulated in response to TNF-α (Pagnotta et al., 2013; M. Yan et al., 2007; Q. H. You et al., 2010), TGF-β, and interleukins 1β and 17F (Q. h You, Sun, Wang, Shen, & Wang, 2010).

<center>*Role of Gravin in Cellular Signaling*</center>

It is likely that gravin mediates its broad physiological impact by binding to a variety of signaling enzymes and targeting them to specific subcellular compartments. The following section will overview the mechanistic role of gravin in cells by outlining its molecular structure, binding partners, and intracellular localization dynamics.

Molecular Structure and Subcellular Distribution

Gravin has a predicted rod-like structure and binding domains for many different molecules (Lin et al., 1996a). Four domains are responsible for plasma membrane localization and include a putative N-terminal myristoylation site and three polybasic domains located at residues 171–191, 296–316 and 507–536. Whereas the putative myristoyl group is added posttranslationally and serves as an N-terminal lipid anchor, gravin's polybasic domains are believed to electrostatically adhere to acidic phospholipids of the plasma membrane (Malbon, Tao, Shumay, & Wang, 2004; Resh, 1999). The polybasic domains also show additional binding affinity for cholesterol, and along with the N-myristoylation site support the notion that gravin may localize preferentially to lipid rafts of the plasma membrane (M. C. Choi, Lee, Kim, Lee, et al., 2008; Su et al., 2013). The relative contribution of each of these domains to plasma

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membrane localization is somewhat complex. Gravin mutants lacking the myristoylation site have seemingly normal localization at the cell periphery, as do gravin mutants that lack the polybasic domains but retain the myristoylation site (Tao, Shumay, McLaughlin, Wang, & Malbon, 2006; X. Yan, Walkiewicz, Carlson, Leiphon, & Grove, 2009). This means that the myristoylation site or all three polybasic domains are each sufficient, but not necessary for membrane localization. The sufficiency of the myristoylation site for membrane localization is surprising given the relatively weak binding energy provided by myristoyl groups – other models suggest that membrane binding due to N-myristoylation requires other membrane-bound binding regions for support (Resh, 1999). The mechanics of gravin membrane localization is important because it plays a central role in regulating the interaction of gravin-anchored enzymes with potential downstream substrates.

Gravin Binds to Protein Kinase A

Several studies suggest that gravin anchors a host of signaling enzymes. Gravin binds to PKA RIIα through C-terminal amphipathic helical domain, a domain which has been shown in other AKAPs to interact with the docking domain (D/D) of RIIα on the hydrophobic pocket of AKAP amphipathic helix (Gold et al., 2006; Nauert et al., 1997). Gravin-PKA interaction directs PKA RII to the cell periphery and is required for the phosphorylation of downstream PKA substrates. Yan et al. (2009) showed that cells expressing both gravin-EGFP and PKA RII-ECFP co-distribute at the cell periphery, and PKA RII-ECFP does not distribute at the cell periphery in cells co-expressing a gravin mutant lacking the PKA binding domain (X. Yan et al., 2009). It is likely that gravin targets PKA to the plasma membrane for phosphorylation of PKA substrates at the plasma membrane. Two known downstream substrates are β2-adrenergic receptor

(β2AR) and gravin itself. Tao et al. (2003) showed that PKA phosphorylates gravin and thereby enhances gravin's association with β2AR, and this phosphorylation does not occur in gravin mutants missing the PKA binding domain. In addition, PKA that is anchored to gravin phosphorylates β2AR and is required for receptor resensitization following isoproterenol induced desensitization (Tao, Wang, & Malbon, 2003). Phosphorylation of additional PKA substrates at the membrane are likely to be regulated by gravin-PKA interaction, but this has not been explored to date.

Gravin Binds to β2-Adrenergic Receptor

Malbon and colleagues have reported extensively on gravin's interaction with β2AR, a G protein-coupled receptor that activates PKA by stimulating cAMP production (G. F. Fan, E. Shumay, H. Y. Wang, & C. C. Malbon, 2001; F. Lin, H. Y. Wang, & C. C. Malbon, 2000; Malbon, Tao, & Wang, 2004; Shih, Lin, Scott, Wang, & Malbon, 1999b; Tao et al., 2006; Tao et al., 2003; Tao, Wang, & Malbon, 2007). Gravin-β2AR binding has been demonstrated in various pull-down assays and is reported to occur between residues 652-938 of gravin and the C-terminal cytoplasmic tail of β2AR (F. Lin, H. Y. Wang, et al., 2000; Shih et al., 1999b; Tao et al., 2003). A thorough study by Tao et al (2003) showed that upon isoproterenol treatment, gravin is phosphorylated on serines 696, 697, 698, and 772 by gravin-anchored PKA, and this phosphorylation strengthens the association of gravin with β2AR. Additionally, gravin-β2AR interaction is required for receptor resensitization following isoproterenol-mediated receptor desensitization (Tao et al., 2003). Knockdown of gravin also prevents the association of β2AR with other molecules like β-arrestin, GRK2, and clathrin, but whether this is dependent on gravin-β2AR interaction has not been demonstrated (F. Lin, H. Y. Wang, et al., 2000). It is

likely that gravin plays a major role not only in regulating β2AR dynamics, but also in influencing other downstream PKA substrates following receptor activation.

Gravin Binds to Protein Kinase C

Gravin has been shown to bind several PKC isoforms in cultured human and rodent cell lines (Grove & Bruchey, 2001; Guo, Gao, Rothschild, Su, & Gelman, 2011; Lin et al., 1996a; Nelson, Moissoglu, Vargas Jr, et al., 1999; Piontek & Brandt, 2003). Gravin-PKC interaction has been reported to suppress ERK1/2 phosphorylation (Su et al., 2010) and also suppress PKC activity itself (Guo et al., 2011), and facilitate phorbol ester-induced cytoskeletal remodeling (Guo et al., 2011). Activation of PKC by phorbol ester triggers the redistribution of gravin to juxtanuclear vesicles and is thought to occur through PKC phosphorylation of gravin at the membrane-binding polybasic domains (Lin et al., 1996a; Malbon, Tao, Shumay, et al., 2004; Nelson, Moissoglu, Vargas Jr, et al., 1999; Piontek & Brandt, 2003; X. Yan et al., 2009). Interestingly, Yan et al. (2009) demonstrated that a mutant gravin-EGFP construct lacking the polybasic domains still underwent redistribution to a juxtanuclear compartment upon PKC activation, which suggests (A) that the mechanism of PKC-mediated gravin redistribution is more complex than phosphorylation of the polybasic domains, and (B) that PKC-mediated gravin redistribution may occur independent of gravin-PKC interaction since this mutant also lacked the putative PKC-binding domain. Interestingly, PKC activation by phorbol ester also causes the upregulation of gravin expression in cultured human erythroleukemia cells (HEL), which do not express gravin under basal conditions (Gordon et al., 1992; Grove et al., 1994; Nauert et al., 1997). Given the findings that PKC is suppressed when

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bound to gravin, it would be interesting to investigate whether this supports a negative feedback loop in PKC-mediated gravin expression levels.

Gravin Binds to Src

Gravin binds to Src within the first fifty amino acids of gravin's N-terminus in human epidermal carcinoma cells, and this interaction is reported to enhance Src activity. It is also required for β2-adrenergic receptor resensitization following isoproterenol induced desensitization (Tao et al., 2007). A more recent study in mouse pancreatic carcinoma cells showed that gravin may also bind to Src at polybasic domain 1, and this interaction strengthens cell adhesion by sequestering Src away from focal adhesion plaques to lipid rafts (Su et al., 2013).

Gravin Binds to Polo-Like Kinase 1

A recent study by Canton et al (2012) elegantly showed that gravin is phosphorylated by CDK1 on threonine 766 during mitosis, and this stimulates the interaction of gravin with Polo-like kinase 1, a mitotic kinase. During mitosis, phospho-gravin and Plk1 congregate alongside nuclear DNA during prophase and at the mitotic spindle during metaphase, but segregate once the cell reaches anaphase. Since gravin knockdown was shown to impact the fidelity of mitotic progression, the authors proposed that this gravin-Plk1 interaction may in part facilitate gravin's role in regulating cell cycle events.

Gravin Binds to Cyclin D1

Gravin has been demonstrated to interact with cyclin D1 in both cultured rodent cells and in mouse parietal epithelial cells of Bowman's capsule (Burnworth et al., 2012; Lin & Gelman, 2002; Yamamoto, Tamakawa, Yoshie, Yaginuma, & Ogawa, 2006). This

interaction occurs through two cyclin binding "CY" motifs between residues 500-517 of rodent gravin and sequesters cyclin D1 away from the nucleus. Interestingly, PKC phosphorylates gravin and inhibits gravin-cyclin interaction, stimulating cyclin D1 to localize back into the nucleus (Burnworth et al., 2012; X. Lin et al., 2000). The authors of these studies propose that gravin regulates G_1-S stalling through its dynamic interaction with cyclin D1.

Gravin Binds to Calmodulin

Two reports highlight the interaction of gravin with calmodulin. In rodent gravin, Lin and Gelman (2002) identified four regions with "1-5-10" consensus sequences for calmodulin binding: two of these sequences (residues 289-297 and 502-511) fall within polybasic domains 2 and 3, and the other two are located downstream between residues 664-673 and 851-860, respectively. Calmodulin bound to peptides containing these four regions in a calcium-dependent manner and was inhibited by PKC activity (Lin & Gelman, 2002). A later study in human gravin also revealed four calmodulin binding domains with some variation to that in rodents: calmodulin bound to polybasic domains 1, 2, and 3 (residues 171-187, 297-317, and 510-536), and a fourth downstream region containing a 1-5-10 motif (residues 670-694) that corresponds to the third calmodulin binding domain of rodent gravin (Tao et al., 2006). It is noteworthy that human gravin lacks the last calmodulin binding motif found in rodent gravin (851-860), and the impact of this is completely unknown. Additionally, it's interesting that human gravin contains only two "1-5-10" motifs at the second and fourth calmodulin binding domain, whereas rodent gravin contains four of these motifs. This suggests that human gravin binds calmodulin at each of these sites with differential affinity, as evidenced in the paper by

Tao et al. (2006) where polybasic domain 2 of human gravin showed greater affinity for calmodulin than did polybasic domains 1 and 3. The affinity of calmodulin for the fourth calmodulin binding domain of human gravin was not explored in Tao's study. The impact of gravin-calmodulin interaction is thought to promote the dissociation of gravin away from the plasma membrane especially through calmodulin interaction with the polybasic domains (Malbon, Tao, Shumay, et al., 2004). Tao et al. (2006) demonstrated that peptides corresponding to polybasic domains 1, 2, and 3 bound to lipid vesicles *in silico*, and this binding was reversed by calmodulin protein in a calcium-dependent manner. This mechanism has also been modeled in other proteins with polybasic domains similar to gravin (McLaughlin, Hangyas-Mihalyne, Zaitseva, & Golebiewska, 2005).

Gravin Binds to Phosphodiesterase Type 4D

Gravin's interaction with phosphodiesterase type 4D (PDE4D) has been demonstrated via pull-down both in human embryonic kidney (HEK293) and cultured rat aortic smooth muscle cells (Raymond, Carter, Ward, & Maurice, 2009; D. Willoughby, W. Wong, J. Schaack, J. D. Scott, & D. M. F. Cooper, 2006b). Willoughby et al. (2006) showed that either inhibition of PKA or disruption of PKA-AKAP interaction inhibited PDE-dependent cAMP hydrolysis at the plasma membrane. Gravin knockdown also inhibited plasma membrane cAMP hydrolysis. Based on these data, gravin was proposed to regulate compartmentalized [cAMP] dynamics by binding to PDE4D and targeting it to the plasma membrane. Importantly, the binding domain for PDE4D on gravin has not been determined, nor has the importance of gravin-anchored PKA been elucidated in gravin's regulation of [cAMP].

Gravin Undergoes Subcellular Redistribution in Response to Calcium and/or PKC Activation.

Although gravin localizes to the plasma membrane under basal conditions, PKC activation or elevation of $[Ca^{2+}]_i$ triggers the redistribution of gravin away from the plasma membrane. With PKC activation, gravin translocates to juxtanuclear vesicles (Lin et al., 1996a; Nelson, Moissoglu, Vargas Jr, et al., 1999; X. Yan et al., 2009). Juxtanuclear relocalization requires the myristoylation site, as mutant constructs missing this N-terminal region translocate from the plasma membrane to the cytosol (X. Yan et al., 2009). Although the mechanics of PKC-mediated gravin redistribution are not completely understood, this event probably occurs through PKC phosphorylation of gravin – which is a known PKC substrate – at sites that are important for membrane localization. PKC phosphorylates gravin peptides containing the membrane-associated polybasic domains (PB1-3) (Lin et al., 1996a), but alternative phosphorylation sites are also likely to be important since a mutant gravin construct missing the polybasic domains also underwent PKC-mediated redistribution from the plasma membrane to juxtanuclear vesicles (X. Yan et al., 2009). Importantly, PKC-mediated gravin redistribution also caused the relocalization of PKA from the plasma membrane to juxtanuclear vesicles, suggesting that gravin facilitates cross-talk between PKC and PKA-dependent signaling pathways.

Calcium elevation was shown by Tao et al. (2006) to cause the redistribution of gravin away from the plasma membrane and into the cytoplasm. Like PKC, the mechanics of calcium-mediated gravin redistribution are also poorly understood, but this event is likely to involve the binding of calmodulin to the membrane-associated polybasic domains (PB1-3). Calmodulin binds to gravin peptides corresponding to polybasic

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domains 1, 2, and 3 in a calcium-dependent manner, releasing these peptides from phospholipid vesicles *in vitro*. Calcium-mediated redistribution of gravin is proposed to regulate β2-adrenergic receptor resensitization following agonist induced desensititzation, but the impact of this event on enzymes bound to gravin is currently unknown. It is likely that through this mechanism, gravin facilitates cross-talk between calcium and PKA-dependent signaling pathways.

<div align="center">Research Goal and Hypothesis</div>

Since gravin's role in cellular signaling depends on the targeting of signaling enzymes to discrete subcellular compartments, stimuli that regulate this subcellular targeting of gravin are also likely to impact the signaling dynamics of a host of enzymes bound to gravin. While it is known that intracellular calcium elevation triggers the redistribution of gravin from the membrane to the cytosol, almost nothing is known about the impact this event might have on signal transduction through signaling enzymes bound to gravin. PKA binding to AKAPs is well characterized, which makes PKA an excellent enzyme of study in investigating the impact of gravin dynamics on cell signaling. Therefore, the *goal* of my research is to determine the impact of calcium mediated gravin redistribution on PKA localization and PKA-dependent signaling.

The central hypothesis of my research is that gravin facilitates crosstalk between calcium and PKA-dependent signaling pathways through subcellular redistribution. To test this hypothesis, I used fluorescent gravin constructs to determine the effect of pharmacological and receptor-mediated calcium elevation on gravin distribution and gravin-directed PKA localization. In addition, I used mutant gravin constructs to explore the role of gravin's calmodulin binding domains in calcium-mediated gravin

redistribution. Finally, I used the FRET-based PKA biosensor AKAR3 to determine the

impact of gravin on subcellular PKA activity under basal conditions and under conditions

which stimulate calcium-mediated gravin redistribution.

CHAPTER II

METHODS AND MATERIALS

Cell Culture and Transfection

Two cell lines were used in this study: AN3 CA cells and HEC-1-A cells (Manassas, VA, ATCC numbers: HTB-111, HTB-112 respectively). Both of these are human adenocarcinoma-derived cell lines from the endometrium of the uterus. AN3 CA cells were derived from a metastatic lesion in the lymph node of a 55 year old patient with endometrial carcinoma. HEC-1-A cells were similarly derived from a 71 year old patient with stage 1A endometrial carcinoma. While AN3 CA cells do not express gravin endogenously, HEC-1-A cells do express gravin sporadically in small clusters of cells (X. Yan et al., 2009).

Cells were maintained at 37°C with 5% CO_2 in low glucose Dubecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and 100 units/ml penicillin and 100ug/ml streptomycin. Growth medium was replaced three times each week, and cells were split 1:25 upon reaching confluence. In experiments where cells were transfected with expression vectors, cells were transfected two to three days after seeding. To perform the transfection, cells were incubated in a transfection solution containing 3µl/ml GeneJammer (Agilent) and 1µg/ml plasmid DNA for twenty-four hours prior to microscopy. For experiments involving co-transfection with AKAR3 and gravin-V5/His constructs, it was critical that cells found expressing AKAR3 also expressed gravin-V5/His. To accomplish this, we found that a 1:3 molar ratio of

AKAR3:gravin plasmid DNA in the transfection mixture yielded transfected cultures in

which roughly 90% of cells expressing AKAR3 also expressed gravin-V5/His (Fig. II-1).

Construction of Expression Vectors

Gravin-EGFP, (ΔPB1-3) gravin-EGFP, and PKA RII-ECFP vectors were initially

constructed as described in previous work (X. Yan et al., 2009). The current study used

several additional gravin constructs which were generated as follows.

Construction of (ΔPKA) Gravin-EYFP
and (ΔPKA) Gravin-V5/His

A gravin-EYFP construct lacking the PKA RII binding domain (nucleotides 4621-

4662) termed "(ΔPKA) gravin-EYFP" was generated in three steps. First, a gravin

fragment containing the PKA binding domain (between AgeI/AgeI restriction sites) was

excised from full length gravin-EYFP and inserted into a pEYFP N1vector. The PKA

binding domain was then deleted from this fragment through a PCR based mutagenesis

approaching using a Phusion® site-directed mutagenesis kit (New England Biolabs,

product F-541) and forward primer and reverse primers

(5'ACAGCCGTTGACCAGTTTGTACGTACAGAA and

5'TTCCAAAATCCCATTTTCAGGCTCTAAATC respectively) flanking the PKA

binding domain. Finally, the mutated AgeI/AgeI gravin fragment generated in the new

vector was substituted for the wild type fragment in the original vector. Cloning of the

gravin fragment into an intermediate plasmid was necessary to due to difficulties

obtaining PCR products from full length gravin. The deletion of the PKA binding domain

was confirmed by sequence analysis. A gravin-V5/His construct missing the PKA-

binding domain – termed "(ΔPKA) gravin-V5/His" – was generated by replacing a DNA

fragment between two XbaI restriction sites of full-length gravin-V5/His with that of

(ΔPKA) gravin-EYFP.

<center>*Construction of (ΔPB1-3, CB4) Gravin-EGFP,*
(ΔPB1-3+) Gravin-EGFP, and (ΔCB4) Gravin-EGFP</center>

A gravin-EGFP vector lacking the three polybasic domains and the fourth

downstream putative calmodulin binding domain (nucleotides 125-2086) termed "(ΔPB1-

3, CB4) gravin-EGFP" was generated by inserting a SacII site directly downstream of the

CB4 domain, and then removing nucleotides 125-2086 by SacII digestion

(Primers:5'GCAAGGAGAAGT[CCGCGG]CTGATGAGGAA ;

5'GGTGCGTCAAAGTCGGCTACGGGGGTGC; underlined nucleotides indicate the

position of the SacII site). A gravin-EGFP vector lacking the polybasic domains and all

nucleotides up to the beginning of the CB4 domain (Δ125-2011) termed "(ΔPB1-3+)

gravin-EGFP" was similarly generated by inserting a SacII restriction site directly

upstream of the CB4 domain, and removing nucleotides 125-2011 by SacII digestion

(Primers:5'CCAAAGC[CCGCGG]TGGATACCTCAGTATCT ;

5'GGTGCGTCAAAGTCGGCTACGGGGGTGC, mutagenic nucleotides underlined,

constructed SacII sequence in brackets). Finally, a gravin-EGFP construct lacking only

CB4 (Δ2005-2079) termed "(ΔCB4) gravin-EGFP" was generated by site-directed

mutagenesis (Phusion®, New England Biolabs, product F-541; forward primer

5'AGGTCGTCTTCTGATGAGGAAGGGGGACCA; reverse primer

5'TGGTTCTTCCGGCTTTGGCTCTTCAACGCT). Like the ΔPKA gravin construct

described above, the ΔCB4 mutation was done in an intermediate vector generated by

inserting a gravin fragment that included the CB4 domain (between AccI-SbfI) into

pmCherry N1. Following PCR mutagenesis and ligation of this intermediate vector, the

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mutated AccI-SbfI fragment missing the CB4 domain was substituted back into the original gravin-EGFP vector. The deletion of the CB4 domain was subsequently confirmed by restriction digest and sequence analysis.

Construction of (mutCB4) Gravin-EGFP
and (mutCB4) Gravin-V5/His

A gravin-EGFP construct with mutations in the CB4 domain – termed "(mutCB4) gravin-EGFP" – was created by site-directed mutagenesis (Phusion®, New England Biolabs, product F-541; forward primer 5'<u>C</u>ATCTTGGGAAGCT<u>GC</u>AATTTGTGTG; reverse primer 5'CTGAGGTATCC<u>G</u>CCTT<u>T</u>CGCTTTGGT; mutagenic nucleotides underlined) resulting in the following mutationsV672A, V676A, and L681A. The mutation indicated at the T position in the reverse primer is a silent mutation introduced to optimize primer design. Site-directed mutagenesis of the CB4 region was performed on the intermediate vector containing the gravin fragment described in the previous paragraph. Following PCR mutagenesis and ligation of this intermediate vector, the mutated Acc1-SbfI gravin fragment was substituted back in to full-length gravin-EGFP. A gravin-V5/His construct with mutations in the in the CB4 domain – termed "(mutCB4) gravin-V5/His" – was constructed by substituting a DNA fragment located between the SacII and EcoRV restriction sites in the full-length gravin-V5/His with a corresponding fragment from the (mutCB4) gravin-EGFP construct.

Construction of Lck-AKAR3

AKAR3 (A-Kinase Activity Reporter 3) is a live-cell biosensor that uses Förster's resonance energy transfer (FRET) to record changes in PKA activity in real time. Two of the AKAR3 biosensors used in this study, AKAR3-NES and AKAR3-CAAX (also called pmAKAR3), were kind gifts of Dr. Jin Zhang (Johns Hopkins) and were constructed as

described previously (Allen & Zhang, 2006). The Lck-AKAR3 construct used in this study was made in our lab by inserting a linker between the HindIII and BamHI restriction sites of AKAR3-NES (Oligos: 5'AGCTTGCCACCATGGGCTGTGTCTGCAGCTCAAACCCTGAAAAG; 5' GATCCTTTTCAGGGTTTGAGCTGCAGACACAGCCCATGGTGGCA).

Western Blotting

To obtain protein samples for Western blotting, AN3 CA cells were transfected in T-25 flasks with 15 µL Genejammer (Aligent) and 5 µg of plasmid DNA per flask. Twenty-four hours later, cells were harvested by scraping into 2 ml of ice-cold PBS (58 mM Na_2HPO_4, 17 mM NaH_2PO_4–H_2O, 68 mM NaCl), pelleted by centrifugation, and resuspended in 50 µl of lysis buffer (20 mM Tris base, 150 mM NaCl, 10 mM EDTA, 10 mM Benzamidine HCl, 1% (v/v) Triton X-100, 0.05% (v/v), Tween 20, 1 mM PMSF and 100 µg/ml leupeptin). Cell lysates were then run on 5% SDS-polyacrylamide gels and transferred to nitrocellulose membranes by standard methods (Towbin, Staehelin, & Gordon, 1979). Blots were then rinsed overnight in blocking buffer (1x PBS, 0.2% I-Block[TM] Reagent, 0.1% Tween®-20 Detergent) and probed using an antigravin polyclonal antibody at a concentration of 1:5,000 for 60 minutes (Grove et al., 1994; Grove & Bruchey, 2001). Blots were then labeled with an alkaline phosphatase conjugate secondary antibody at a concentration of 1:100,000 for 60 minutes, and immunoreactive bands were detected by incubating blots in CDB Star chemiluminescence reagent.

Immunofluorescence Labeling of Cultured Cells

For immunofluorescence labeling, cells were fixed in 3.7% paraformaldehyde in PBS (150 mM NaCl, 4 mM Na/K phosphate buffer and 5 mM KCl) for 10 minutes and

rinsed three times with PBS. Cells were then permeabilized with digitonin for 10

minutes, rinsed with PBS, and treated for 30 minutes with 5% normal goat serum at room

temperature to block non-specific binding of the antibody. Next, cells were treated with

an anti-gravin monoclonal mouse antibody at a concentration of 1:1000 for 60 minutes at

37° C, as previously described (X. Yan et al., 2009). Primary antibody labeling was

detected using CY3 conjugated donkey anti-mouse secondary antibody (Jackson

Immunoresearch, Inc., West Grove, PA) at a concentration of 1:200 for 60 minutes at 37°

C. Cells were mounted to glass slides using Pro-long® Gold antifade reagent with DAPI

(Life Technologies, P36935).

Epifluorescence Microscopy

Nikon TE300 Microscope

Experiments using quantitative imaging (calcium dynamics, gravin redistribution,

FRET microscopy) were done on a Nikon TE300 inverted microscope equipped with a

Hamamatsu camera, Ludl excitation and emission filter wheels, and a range filters for

fluorescence microscopy. This microscope uses a mercury lamp to generate fluorescence

excitation, which is passed through a combination of neutral density filters that control

the intensity of excitation from the mercury lamp. Neutral density filter settings were

optimized in each experiment to minimize photobleaching of fluorescent proteins.

Various excitation filters allowed only certain wavelengths of light to pass to the sample,

and these filters were located either in an automated excitation filter wheel or within one

of four filter cubes mounted on a slider underneath the objective lens carousel. Excitation

light passes through these excitation filters and is reflected up to the sample through and

objective lens by a dichroic mirror within one of the filter cubes. As the sample is

excited, its emission fluorescence is passed back down through the objective lens, and the same dichroic which reflected the excitation light up toward the sample also allows emission fluorescence to pass back through the mirror where it is reflected either to the ocular eyepieces or to a Hamamatsu CCD camera. (Fig. II-2) Emission filters allow only certain wavelengths of emission fluorescence to reach the camera, and these emission filters are situated either in the automated emission filter wheel or within the filter cubes.

Images were acquired using software called Micro-Manager, an open source microscope controller developed by Vale and colleagues (Edelstein, Amodaj, Hoover, Vale, & Stuurman, 2010). Micro-manager was used to control both the excitation and emission filter wheels, the camera sensitivity (gain), and exposure time which was controlled by a shutter in the excitation filter wheel. In a time-course experiment, a set number images could be collected using multiple excitation/emission filter settings at a specified time-interval. Image quantification was subsequently performed using ImageJ software.

Calcium Imaging

Changes in $[Ca^{2+}]_i$ were measured with Fura-2 using the ratio of fluorescence intensity at excitation wavelengths of 340 and 380 nm. Cells on coverslips were incubated at 37°C with 1 mg/ml Fura-2 AM (Invitrogen/Molecular Probes) for 30 minutes, rinsed with Standard Extracellular Solution (SES), and placed in an Attofluor chamber heated to 37º C containing either SES (1.5 mM $CaCl_2$ dihydrate, 145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$ anhydrous, 10 mM glucose, 10 mM Hepes) or Ca^{2+} free SES (145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$ anhydrous, 10 mM glucose, 10 mM Hepes). Images were obtained at 15 or 30 second intervals using a 40X Oil Plan Fluor objective

lens (NA=1.3) mounted on a Nikon TE300 inverted microscope, and acquisition settings

were the same for each experiment (See Fig. II-3). Following acquisition, Fura-2

fluorescence images were background subtracted, and the ratio of Fura-2 fluorescence

intensity at 340 and 380 nm excitation wavelengths was measured for 10-20 cells per

coverslip at each time interval using ImageJ plugins for background subtraction and

ratiometric analysis (compiled by Tony Collins, McMaster Biophotonics Facility,

Department of Biochemistry and Biomedical Sciences, McMaster University, Hamilton,

ON).

Quantification of Gravin Redistribution

Changes in the subcellular distribution of wild type or mutant gravin-EGFP

following agonist treatment were quantified by calculating the ratio of membrane to

cytosolic fluorescence intensity from images acquired at 15 or 30 second intervals using

the Nikon TE300 inverted microscope. Acquisition parameters were the same for each

experiment (See Fig. II-4). Following background subtraction in ImageJ, this ratio was

generated by measuring the fluorescence intensity profile along a line 1 pixel wide by 36

pixels long drawn at several points across the plasma membrane of cells expressing

gravin-EGFP (Fig. II-5A), calculating the mean fluorescence intensity of the four

brightest pixels at the peak of the intensity profile and dividing that by the mean

fluorescence intensity of the profile corresponding to the cytosolic portion of the cell

(Fig. II-5B,C). Preliminary analysis of cells in which the plasma membrane was labeled

with FM4-64 revealed that the plasma membrane region of the line profile was marked

by a sharp elevation in intensity roughly 4 pixels in width, while the region of the line

profile on the cytosolic side of these peak values provided a reasonable representation of

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the cytosolic fluorescence intensity. Points of measurement across the plasma membrane were carefully selected to avoid non-fluorescent intracellular regions (e.g. nucleus) and brightly fluorescent puncta within the cytosol. Loss of gravin-EGFP at the cell cortex resulted in a decrease in membrane fluorescence intensity, an increase in cytosolic fluorescence intensity, and a decrease in the ratio of membrane fluorescence against cytosolic fluorescence (Fig II-5D). Ratios were normalized by dividing each ratio in the time series by the mean ratio prior to agonist treatment. All analysis and quantification was done on images in their native format (16-bit, 72 pixels/inch). For figures, cropped greyscale images of gravin-EGFP were converted to 8-bit and resized using Adobe Photoshop.

Quantification of Gravin Localization at the Cell Periphery

Assessment of gravin localization at the membrane was done by imaging cells at predefined XY locations on coverslips, as set by a grid positioned on the microscope stage. The center of each coverslip corresponded to the center of the grid, and images were collected within nine predefined regions (Fig. II-6). Images were placed in random order, and each cell was scored as "yes" or "no" for gravin localization at the cell periphery in a blinded manner. The mean percentage of cells showing localized gravin at the cell periphery was then calculated for each coverslip.

Quantification of FRET Biosensor Dynamics

Prior to each experiment, AKAR3 transfected AN3 CA cells plated on 25 mm coverslips were incubated at 37°C for 30 minutes in standard extracellular solution (SES; 1.5 mM $CaCl_2$ dihydrate, 145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$ anhydrous, 10 mM glucose, 10 mM Hepes, pH 7.3) containing 10 μM IBMX (3-isobutyl-1-methylxanthine;

Sigma I-7018). Dual CFP/FRET images of the cells were acquired at 30-second intervals using the Nikon TE300 inverted microscope and the 40x/1.3NA Oil Plan Fluor objective lens. Filters used consisted of a 440AF21 excitation filter, a 455DRLP dichroic mirror and two emission filters (480AF30 for CFP and 535AF26 for FRET) mounted in a filter wheel. Excitation light intensity was adjusted using neutral density filters to minimize photobleaching. Acquisition parameters were the same for each experiment (Fig. II-7).

Confocal Microscopy

Live-cell imaging of cells co-transfected with gravin-EYFP and PKA RII-ECFP were obtained using a Zeiss 510 META laser scanning confocal microscope, with a Zeiss 100x Plan-Fluar oil objective lens (NA 1.45). Cells were incubated in regular SES medium and maintained at 37°C throughout each experiment. Two color imaging was performed to simultaneously capture EYFP and ECFP fluorescence: EYFP excitation light was generated with a 514 nm argon laser operated at 10% power, and ECFP was excited by a 458 nm argon laser at 80% power. Fluorescence emissions were passed through a NFT 515 beam splitter; 475 nm ECFP longpass filter and 530 nm EYFP longpass filter. Because control images of cells expressing gravin-EYFP or PKA RII-ECFP alone were critical in demonstrating minimal signal crossover, detector gain and amplifier offset settings were changed only slightly (EYFP channel: 825-900 gain, -0.18 to 0.01 offset; ECFP channel: 1150-1250 gain, -0.13 to -0.01 offset). Post-acquisition adjustments to image brightness and contrast were applied equally across corresponding images from different experimental treatments.

Table II-1. List of reagents used in this study.

Name	Abbreviation	Source	Catalog #	Description	Stock Conc.	Working Conc.
1,2-Bis (2-aminopheoxy) Ethane-N,N,N',N'-tetraacetic acid tetrakis (acetoxymethylester)	BAPTA-AM	Calbiochem	196419	Calcium chealator	50 mM (DMSO)	10 μM
3-Isobuty1-1-methylxanthine	IBMX	Sigma	I-7018	Inhibits cAMP hydrolysis, inhibits phosphodiesterases	100 mM (DMSO)	10 μM
Adenosine 5'-triphosphate disodium salt hydrate	ATP	Sigma	A2383	Purinergic receptor agonist	Solid	100 mM
Alkaline phosphatase conjugate				Secondary antibody for Western blot		1:100,000
Bisindolylmaleamide	BIM	Calbiochem	203290	PKC inhibitor	2mM (DMSO)	20 μM
Bovine Serum Albumin	BSA	Sigma	A-7030	Used to block non-specific antibody binding	Solid	0.1% in 1xPBS
CY3 2° antibody	CY3	Jackson Immunoresearch	715-165-150	Donkey anti-mouse	1.4 mg/ml	1:200
Digitonin		Sigma	D5628	Permeabilizes cell membranes for IF antibody labeling	50 mg/ml	6.2 μl/ 10 ml in 1x PBS
Forskolin	Fsk	Sigma	F6886	cAMP elevation, stimulates adenylyl cyclase	100 mM (DMSO)	10 μM
Gravin 1° monoclonal antibody	2B3-1.1			Mouse antibody for immunofluorescence	Ascites (mouse)	1:1,000
Gravin 1° polyclonal antibody	Rb7753			Rabbit antibody for Western blot	Serum (rabbit)	1:5,000
I-Block™ Reagent		Tropix	A1300	Blocking buffer used in Western blot	Solid	.02%
Ionomycin	IM	Calbiochem	407952	Calcium elevator	2 mM (DMSO)	1 μM
Normal Goat Serum	NGS	Thermo Scientific	31873	Used to block non-specific antibody labeling	60 mg/ml	5% or 1%
Paraformaldehyde		Polysciences, Inc.	00380	Fixes cells for IF labeling	Solid	3.7%
Thapsigargin	Tg	Sigma	T9033	Calcium elevator	10 mM (DMSO)	1 μM, 10 μM
Tropix® CDP Star®		Applied Biosystems	T2218	Chemiluminescence reagent for Western blot		
Tween® detergent		Sigma	P7949	Used in some Western blot buffers		0.1%

Table II-2. Expression vectors created for this study.

Name	Type	Nucleotides	Amino Acids	Method	Primers
(ΔPKA) Gravin-EGFP	Deletion	Δ4621–4662	Δ1541-1554	Site-directed mutagenesis	5'ACAGCGCGTTGACCAGTTTGTACGTACAGAA 5'TTCCAAAATCCCATTTTCAGGCTCTAAATC
(ΔPKA) Gravin-V5/His	Deletion	Δ4621–4662	Δ1541-1554	Fragment swap	
(ΔCB4) Gravin-EGFP	Deletion	Δ2005–2079	667-693	Site-directed mutagenesis	5'AGGTCGTCTTCTGATGAGGAAGGGGGACCA 5'TGGTTCTTCCGGCTTTGGCTCTTCAACGCT
(ΔPB1-3, CB4) Gravin-EGFP	Deletion	Δ125–2086	42-696	SacII site insert, then SacII digest	5'CCAAAGC[CCGCGG]TGGATACCTCAGTATCT 5'GGTGCGTCAAAGTCGGCTACGGGGGTGC
(ΔPB1-3+) Gravin-EGFP	Deletion	Δ125–2011	42-671	SacII site insert, then SacII digest	5'GCAAGGAGAAGT[CCGCGG]CTGATGAGGAA 5'GGTGCGTCAAAGTCGGCTACGGGGGTGC
(mutCB4) Gravin-EGFP	Mutation	See primers	V673A V677A L682A	Site-directed mutagenesis	5'CATCTTGGGAAGCTGCAATTTGTGTG 5'CTGAGGTATCCGCCTTTCGCTTTGGT
(mutCB4) Gravin-V5/His	Mutation			Fragment swap	
Lck-AKAR3	Addition			Oligonucleotide lnker (between HindIII and BamHI)	5'AGCTTGCCACCATGGGCTGTGTCTGCAGCTCAAACCCTGAAAAG; 5'GATCCTTTTCAGGGTTTGAGCTGCAGACACAGCCCATGGTGGCA
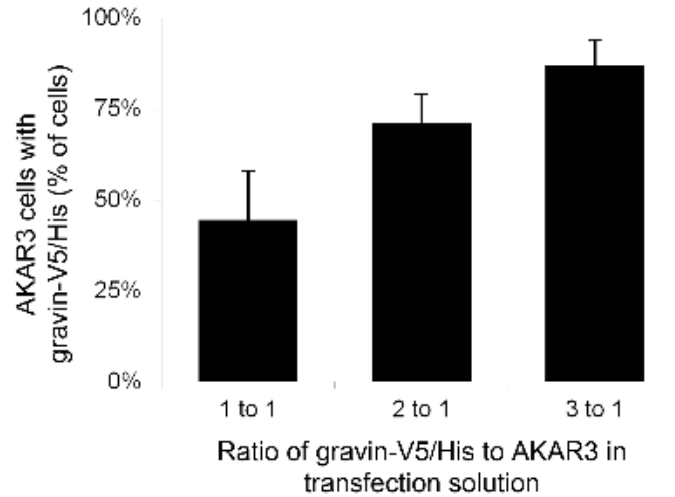
Figure II-1.   Optimization of transfection conditions for co-expression of AKAR3 and non-fluorescent gravin-V5/His constructs. Plasmid DNA of gravin-V5/His and AKAR3-CAAX were mixed at different microgram ratios prior to transfection while maintaining a constant concentration of 1µg of DNA per 1 ml of growth medium. After transfection, cells expressing gravin were detected by immunofluorescence labeling with a gravin primary antibody and CY3 secondary antibody and imaged through a CY3 fluorescence channel. Cells expressing AKAR3-CAAX were detected using a CFP channel. Cell counts on multiple coverslips were performed to determine the percentage of AKAR3 transfected cells which were also transfected with gravin-V5/His. As seen in the graph, the highest percentage was seen when the gravin:AKAR3 microgram ratio of 3:1 was used. This was used for all subsequent experiments in which V5/His tagged gravin constructs and AKAR3 constructs were used together.
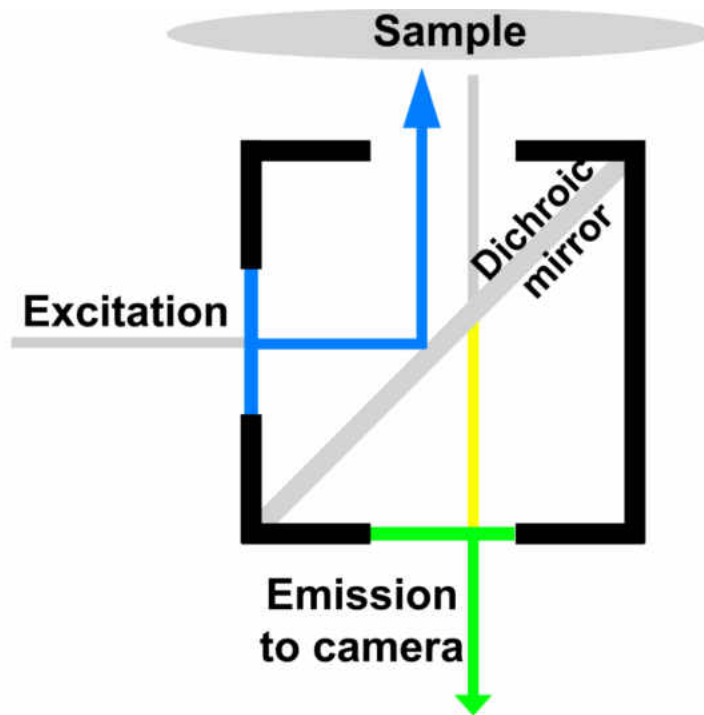
Figure II-2.   Schematic diagram of filter cubes used on the Nikon TE300 microscope.
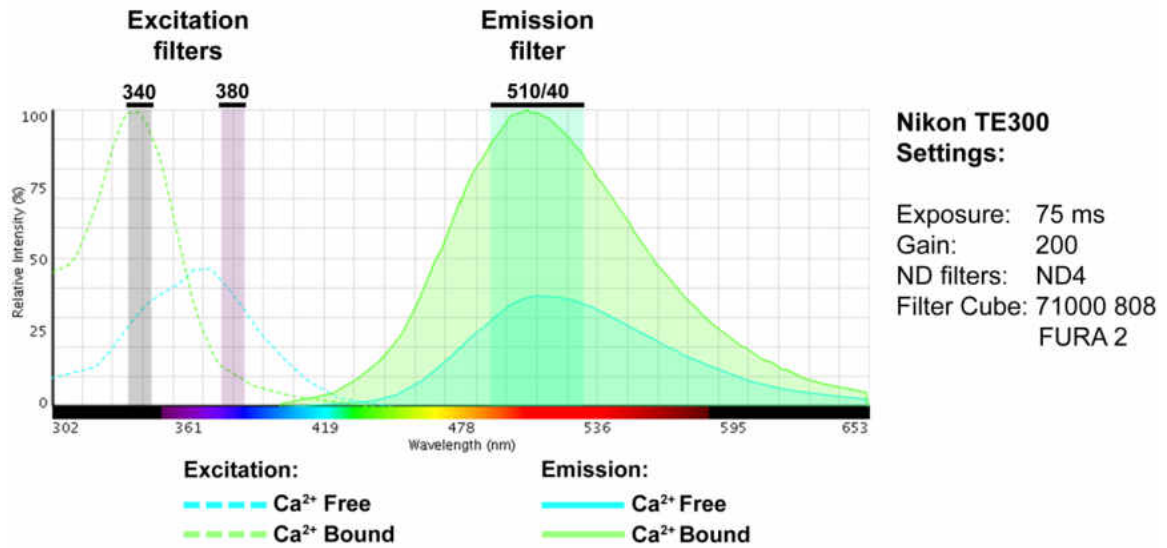
Figure II-3.   Filters and acquisition settings for Fura-2 fluorescence imaging on the Nikon TE300 microscope.
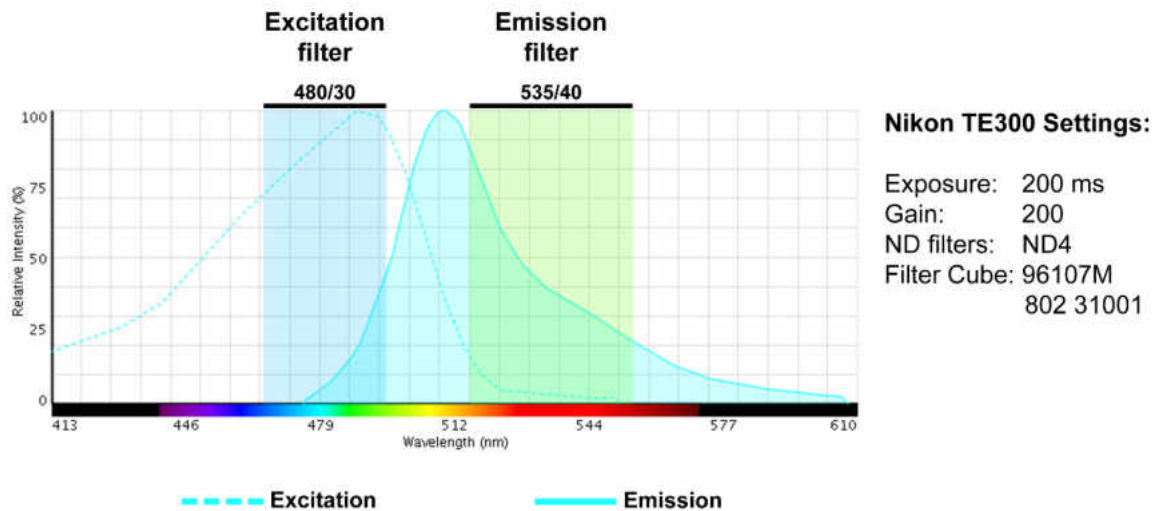
Figure II-4.   Filters and acquisition settings for EGFP fluorescence imaging on the Nikon TE300 microscope.
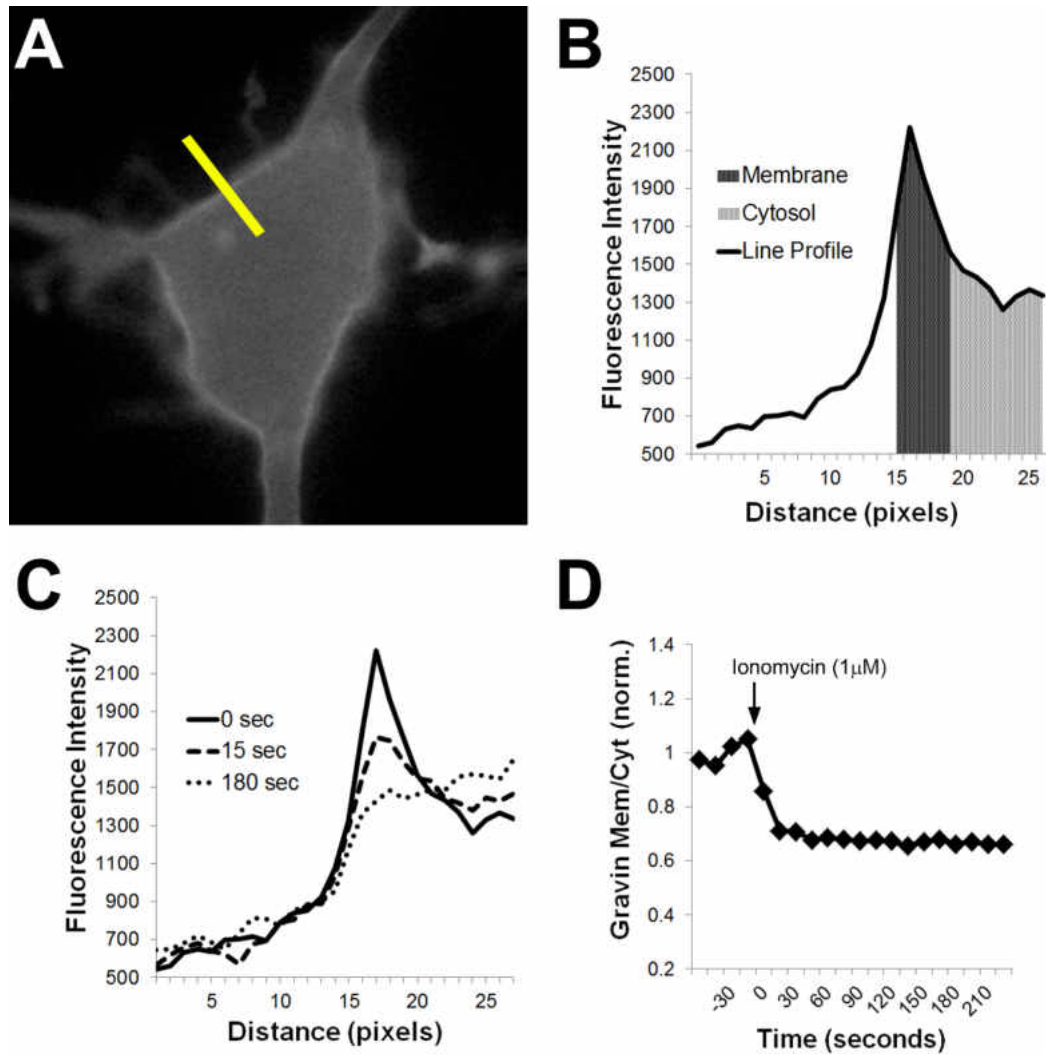
Figure II-5.   Quantification of gravin-EGFP redistribution. This was accomplished by measuring fluorescence intensity along a line transect across the plasma membrane using ImageJ (A). When gravin-EGFP was localized to the cell cortex, the fluorescence intensity along this line yielded a sharp peak in intensity at the membrane with cytosolic intensity following the peak. However, as gravin-EGFP underwent redistribution away from the membrane and into the cytosol, the membrane intensity decreased while the cytosolic intensity increased (B). The average fluorescence intensity at the membrane and at the cytosol was determined for each time point (C) and plotted as a ratio of membrane to cytosolic intensity over time (D). Ratios of membrane to cytosolic intensity (Mem/Cyt) were normalized by dividing each ratio in the time series by the average ratio value prior to treatment.

12mm Coverslip
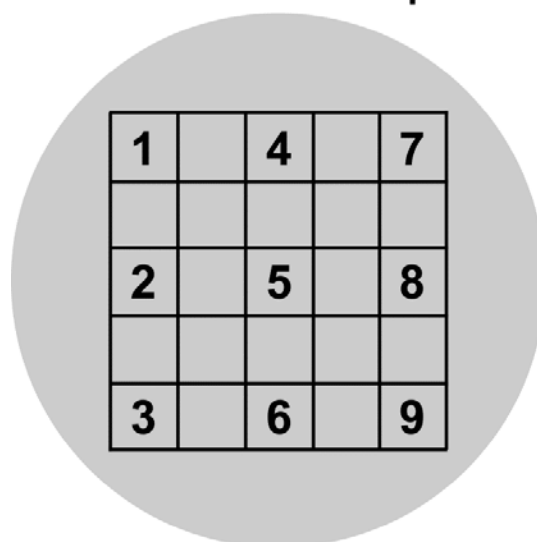
Figure II-6.   Quantification of gravin localization at the cell periphery. Nine images were acquired per coverslip at these pre-defined locations on a grid that was mounted to the stage of the Nikon TE300 microscope.

CFP/YFP FRET Fluorescence
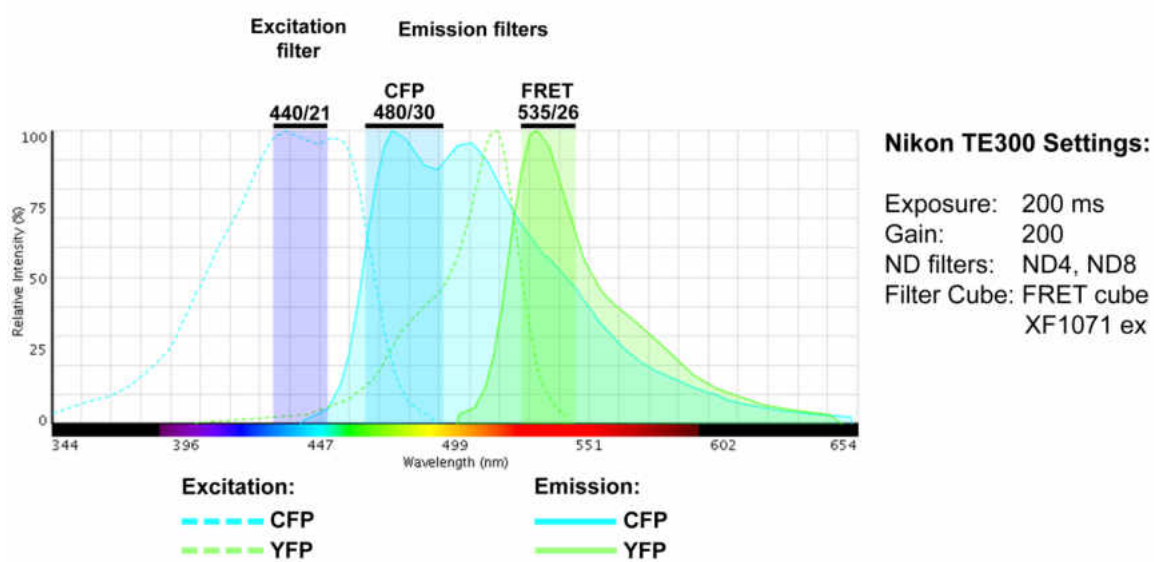
Figure II-7.   Filters and acquisition settings for FRET and CFP fluorescence imaging on the Nikon TE300 microscope.

CHAPTER III

RECEPTOR-MEDIATED Ca^{2+} AND PKC SIGNALING TRIGGERS THE LOSS OF
CORTICAL PKA COMPARTMENTALIZATION THROUGH THE
REDISTRIBUTION OF GRAVIN

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Abstract

A-Kinase Anchoring Proteins (AKAPs) direct the flow of cellular information by positioning multiprotein signaling complexes into proximity with effector proteins. However, certain AKAPs are not stationary but can undergo spatiotemporal redistribution in response to stimuli. Gravin, a 300kD AKAP that intersects with a diverse signaling array, is localized to the plasma membrane but has been shown to translocate to the cytosol following the elevation of intracellular calcium ($[Ca^{2+}]_i$). Despite the potential for gravin redistribution to impact multiple signaling pathways, the dynamics of this event remain poorly understood. In this study, quantitative microscopy of cells expressing gravin-EGFP revealed that Ca^{2+} elevation caused the complete translocation of gravin from the cell cortex to the cytosol in as little as 60 seconds of treatment with ionomycin or thapsigargin. In addition, receptor mediated signaling was also shown to cause gravin redistribution following ATP treatment, and this event required both $[Ca^{2+}]_i$ elevation and PKC activation. To understand the mechanism for Ca^{2+} mediated gravin dynamics, deletion of calmodulin-binding domains revealed that a fourth putative calmodulin binding domain called CB4 (a.a. 670-694) is critical for targeting gravin to the cell cortex despite its location downstream of gravin's membrane-targeting domains, which include an N-terminal myristoylation site and three polybasic domains. Finally, confocal microscopy of cells co-transfected with gravin-EYFP and PKA RII-ECFP revealed that gravin redistribution mediated by ionomycin, thapsigargin, and ATP each triggered the gravin-dependent loss of PKA localized at the cell cortex. Our results support the hypothesis that gravin redistribution regulates cross-talk between PKA-dependent signaling and receptor-mediated events involving Ca^{2+} and PKC.

Introduction

Compartmentalization of intracellular signaling molecules is essential to signal transduction and is achieved in part by scaffold proteins, which have the ability to shape and direct intracellular signaling networks by organizing molecular complexes into distinct subcellular compartments (Good et al., 2011). The ability of scaffold proteins to control the flow of signal transduction in space and time gives them a powerful role in regulating cellular behavior. Conceptually, scaffold proteins help provide a framework for understanding how sophisticated signaling networks can function accurately and efficiently among a widely diverse milieu of intracellular signaling proteins, some with opposing effects. PKA, a critical intracellular enzyme with several hundred phosphorylation substrates (Gao et al., 2008), relies heavily upon spatial targeting to exert its signaling effects. This occurs largely through A-Kinase Anchoring Proteins (AKAPs), which anchor PKA to various subcellular locations through a conserved amphipathic helical domain and a widely varied subcellular targeting domain. AKAPs are known to localize to a number of cellular compartments including the cytoskeleton, plasma membrane, nucleus, Golgi, and endoplasmic reticulum, positioning multiprotein signaling complexes into proximity with other effector proteins (reviewed in Welch et al., 2010). Numerous reports demonstrate that the loss of PKA compartmentalization significantly disrupts PKA signaling and leads to a many physiological dysfunctions, for example, in memory (Nijholt et al., 2008), immune response (Schillace et al., 2009), and cytoskeletal dynamics (Y. Wang et al., 2006).

The AKAP12 gene encodes a 300kD AKAP known as gravin in humans (Gordon et al., 1992; Nauert et al., 1997) or SSeCKS (Lin, Tombler, Nelson, Ross, & Gelman,

1996b) in rodents, that binds the RII subunit of PKA and is expressed in a variety of cell types and tissues (Grove et al., 1994). Three isoforms of gravin have been described (gravin-α, -β, -γ) which differ in subcellular localization (Streb et al., 2004). The canonical α isoform used in this study is expressed in humans and is localized to the cell cortex through an N-myristoylation sequence and by three polybasic domains (PB1-3) which adhere to negatively charged phospholipids (Tao et al., 2006; X. Yan et al., 2009). Like many AKAPs, gravin is a multivalent scaffold and interacts not only with PKA, but also with PKC (Grove & Bruchey, 2001; Nauert et al., 1997; Piontek & Brandt, 2003), phosphodiesterase 4D (D. Willoughby, W. Wong, J. Schaack, J. D. Scott, & D. M. Cooper, 2006a), Ca^{2+}/calmodulin (Tao et al., 2006), and the β2-adrenergic receptor (G. Fan, E. Shumay, H. Wang, & C. C. Malbon, 2001; F. Lin, H. Wang, & C. C. Malbon, 2000; Shih, Lin, Scott, Wang, & Malbon, 1999a; Tao et al., 2003). Accordingly, gravin has been implicated in a wide range of cellular functions. Gelman and colleagues have published extensively on the role of SSeCKS as a tumor suppressor (Akakura et al., 2008; Gelman, 2010; Gelman, Lee, Tombler, Gordon, & Lin, 1998; Gelman et al., 2000). Along similar lines, Choi et al. (M. C. Choi, Lee, Kim, Park, et al., 2008) demonstrated in human SNU-449 heptaocellular cancer cells that gravin is critical in cytokinesis and interacts with actin and myosin light chain kinase during cell division. More recently, Scott and colleagues showed that gravin recruits Polo-like kinase to the mitotic spindle to regulate cell cycle progression (D. A. Canton et al., 2012). Malbon and colleagues determined that gravin associates with the β2-adrenergic receptor in A293 cells and is crucial in regulating this receptor's desensitization and resensitization (G. Fan et al., 2001; F. Lin, H. Wang, et al., 2000; Shih et al., 1999a; Tao et al., 2003). Willoughby et

al. (Willoughby et al., 2006a) determined that gravin-anchored PKA and PDE4D establish a negative feedback loop for regulating [cAMP] in the submembrane compartment of HEK293 cells. It is clear from these examples that gravin intersects with a broad network of signaling pathways; however the precise molecular dynamics behind these diverse functions remain poorly understood.

Although gravin (α isoform) localizes at the plasma membrane under basal conditions, it is known to translocate to alternative subcellular compartments in response to stimuli. Further knowledge of this may be critical in understanding how gravin regulates the activity of its binding partners. Redistribution of gravin from the cell periphery to the cytosol has been demonstrated in response to PKC activation by phorbol ester treatment (Nelson, Moissoglu, Vargas, Klotman, & Gelman, 1999; Piontek & Brandt, 2003). Previous work in our lab demonstrated that PKC activity directs gravin to a vesicular compartment near the nucleus, and that this translocation also causes similar redistribution of PKA (X. Yan et al., 2009). In addition, elevation of intracellular calcium concentration ($[Ca^{2+}]_i$) has been shown to cause gravin redistribution through a presumed mechanism involving Ca^{2+}/calmodulin (CaM) binding to gravin's membrane-associated polybasic domains (PB1, 2 and 3) (Tao et al., 2006). Although CaM binding to PB1-3 has been clearly demonstrated, the notion that this interaction is alone responsible for Ca^{2+} mediated gravin redistribution may be incomplete in light of our previous findings which show that the myristoylation site is sufficient to target gravin to the plasma membrane even in the absence of PB1-3 (X. Yan et al., 2009). While it is likely that CaM binding to PB1-3 contributes to the dissociation of gravin from the plasma membrane, the role of the myristoylation sequence in the event is yet to be determined. In addition, it is currently

unknown whether calcium mediated gravin redistribution also results in the redistribution

of PKA, or if these dynamics are linked to receptor-mediated signaling. Given that PKA

signaling often relies heavily on spatial compartmentalization, the findings that both

$[Ca^{2+}]$ elevation and PKC activation lead to gravin redistribution raise the interesting

possibility that signaling cascades involving Ca^{2+} and/or PKC may engage in cross-talk

with PKA-dependent signaling events through the redistribution of gravin. This would be

a novel finding particularly with regard to Ca^{2+}/PKA crosstalk, which has been thought to

occur primarily through Ca^{2+} sensitive adenylyl cyclases and phosphodiesterases which

regulate cAMP concentrations. From these observations, we hypothesize that gravin

redistribution is mediated by receptors which trigger $[Ca^{2+}]_i$ elevation and/or PKC

activation, and that these dynamics also cause the redistribution of PKA.

To test the hypothesis that calcium-mediated gravin redistribution also triggers

PKA redistribution, we used fluorescent constructs of gravin and PKA RII to determine

the effect of cytosolic calcium increase on gravin-PKA localization. Our results revealed

that both gravin and PKA are redistributed away from the cell periphery following

extracellular calcium influx, release of calcium from intracellular stores, or upon

activation of purinergic receptors by ATP. In addition, these studies demonstrate that

purinergic P2Y receptors utilize both PKC activation and $[Ca^{2+}]_i$ increase to trigger

gravin-PKA redistribution. Although calcium-mediated gravin redistribution has been

proposed to occur through CaM binding to polybasic regions 1-3, our studies additionally

demonstrate that the deletion of these regions had no effect on gravin redistribution. A

fourth calmodulin binding domain, termed CB4, was also assessed to determine the role

of calmodulin binding on gravin redistribution; however deletion of this region dramatically reduced the membrane localization of gravin.

Results

Ionomycin and Thapsigargin Cause the Calcium-Dependent Redistribution of Gravin

Treatment of cells expressing gravin-EGFP with the calcium ionophore ionomycin (1μM) resulted in the complete translocation of gravin-EGFP from the cell cortex to the cytosol (Fig. III-1A images). Plots of the ratio of membrane to cytosolic fluorescence over time revealed that gravin redistribution began immediately after ionomycin treatment and was complete within 60 seconds, at which time $[Ca^{2+}]_i$ elevation reached its plateau (Fig. III-1B,C).

To determine if ionomycin mediated redistribution of gravin-EGFP was indeed calcium-dependent, inhibition studies were performed in regular and Ca^{2+} free SES medium by treating cells with BAPTA-AM (10 μM), a high-affinity intracellular calcium chelator, for 30 min prior to ionomycin treatment. In regular SES medium, control ionomycin treatments with no BAPTA-AM resulted in the complete redistribution of gravin and an immediate, sustained increase in $[Ca^{2+}]_i$ (Fig. III-2A,B). In Ca^{2+} free SES, ionomycin caused both the increase of $[Ca^{2+}]_i$ and the redistribution of gravin, although the magnitude and duration of calcium increase were substantially reduced (Fig. III-2E,F). This finding is consistent with other studies which have demonstrated that ionomycin targets the release of calcium from intracellular stores in addition to triggering extracellular calcium influx (Morgan & Jacob, 1994; Yoshida & Plant, 1992). Ionomycin mediated gravin redistribution and cytosolic calcium increase were both fully prevented in Ca^{2+} free SES when BAPTA-AM was present (Fig. III-2G,H), confirming that

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ionomycin mediated gravin redistribution is calcium dependent. Surprisingly, pre-treatment of cells with BAPTA-AM in regular SES also showed ionomycin-mediated gravin redistribution and a sustained $[Ca^{2+}]_i$ increase; however there was a 60 second delay in gravin redistribution and $[Ca^{2+}]_i$ increase (Fig. III-2C,D). Even at BAPTA-AM concentrations as high as 500 µM, ionomycin caused gravin redistribution in regular SES (not shown) and suggests that the intracellular BAPTA-AM concentration required to prevent $[Ca^{2+}]$ elevation in these cells could not be achieved. Nonetheless, our finding that BAPTA-AM prevented gravin redistribution in Ca^{2+} free SES demonstrated that ionomycin-mediated gravin redistribution was calcium-dependent.

To examine the effect of $[Ca^{2+}]_i$ increase through the release of Ca^{2+} from intracellular stores on gravin distribution, cells expressing gravin-EGFP were treated with thapsigargin, an agent which rapidly elevates cytosolic calcium by inhibiting SERCA-mediated Ca^{2+} reuptake into the endoplasmic reticulum (Lytton, Westlin, & Hanley, 1991). Upon treatment with thapsigargin (0.4 µM), gravin-EGFP underwent redistribution from the cell periphery to the cytosol in both the presence and absence of extracellular Ca^{2+}. Thapsigargin mediated calcium increase peaked within 60 seconds and then gradually decreased to basal levels over the course of several minutes (Fig. III-3A-B,E-F). Pre-treatment of cells with BAPTA-AM (10 µM) 30 minutes prior to thapsigargin treatment prevented gravin redistribution and cytosolic calcium increase in both regular and Ca^{2+} free SES (Fig. III-3C-D,G-H). These results confirm that thapsigargin elicited gravin redistribution in a calcium-dependent manner, raising the possibility that receptor-mediated signaling events which target the release intracellular Ca^{2+} stores into the cytosol would also cause the redistribution of gravin.

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Role of Putative Calmodulin Binding Domains in Ca^{2+}-Mediated Gravin Redistribution

Currently, the mechanism underlying redistribution of gravin in response to elevated $[Ca^{2+}]_i$ is poorly understood. However, a study by Tao et al. (Tao et al., 2006) proposed that calcium mediated gravin redistribution occurs as a result of Ca^{2+}/calmodulin binding to three polybasic domains located in the N-terminal half of gravin (PB1-3) and inhibiting their association with the plasma membrane (Tao et al., 2006), Based on this, we predicted that deletion of PB1-3 would alter the rate of gravin redistribution in response to ionomycin treatment. However, fluorescence microscopy revealed that a gravin-EGFP construct lacking the three polybasic domains (ΔPB1-3) localized to the cell cortex prior to treatment and underwent redistribution from the cell cortex to the cytosol at an identical rate to the wild type gravin construct after ionomycin treatment (Fig. III-4A). This suggests that the polybasic domains PB1-3 are not required for calcium-mediated redistribution of gravin.

The study by Tao et al. (Tao et al., 2006) also revealed the location of a possible fourth calmodulin binding domain on gravin between amino acids 670-694 which, unlike polybasic domains 1-3, did not bind to phospholipid vesicles. This fourth putative calmodulin binding domain (CB4) conforms to a 1-5-10 consensus sequence for Ca^{2+}/calmodulin binding (Rhoads & Friedberg, 1997) and corresponds exactly to the SSeCKS-3 domain, which was shown to bind calmodulin in SSeCKS, the murine orthologue of gravin (Lin & Gelman, 2002). To further understand the role of all four calmodulin binding domains of gravin in Ca^{2+} mediated redistribution, additional mutant gravin constructs were generated with the intent of comparing their rates of redistribution to that of the full length (WT) gravin. A gravin-EGFP construct lacking the polybasic

domains and everything up to but not including the CB4 domain (ΔPB1-3+) localized to the cell cortex prior to treatment, but in response to ionomycin (1 μM) underwent translocation of away from the cell cortex at a significantly delayed rate compared to WT gravin (Mann-Whitney Rank Sum Test, horizontal bar denotes time points at which $p < 0.05$) (Fig. III-4B). Surprisingly, a gravin-EGFP construct lacking the three polybasic domains and the fourth putative calmodulin binding domain (ΔPB1-3, CB4) did not localize at the cell cortex in a manner which was sufficient to measure its rate of redistribution. As illustrated in Fig. III-4C, 75.7% (± 14.8%, 231 cells) of cells transfected with full-length (WT) gravin-EGFP, displayed cortically localized fluorescence, while only 17.8% (± 11.4%, 215 cells) of cells expressing (ΔPB1-3,CB4) gravin-EGFP displayed any appreciable cortical fluorescence. Normal membrane localization was observed in (ΔPB1-3+) gravin-EGFP (81.7% ±10.2%, 125 cells) compared to WT gravin, but a gravin-EGFP construct lacking only the CB4 domain (ΔCB4) localized to the cell cortex in significantly fewer transfected cells (19.7% ± 6.2%, 90 cells) (ANOVA followed by Holm-Sidok post-hoc, significant differences from WT gravin denoted by asterisks, $p < 0.05$).

The expression of the mutant gravin–EGFP vectors was confirmed in AN3 CA cells by Western blotting using an anti-gravin antibody. As seen in Fig. III-4D, the lanes loaded with lysates from different gravin–EGFP transfectants showed gravin expression at the positions that matched the size of the desired gravin–EGFP mutants. No gravin expression was detected in control untransfected cells. These findings demonstrate that the presence of the CB4 domain is critical in targeting gravin to the cell cortex, and that this domain seems to regulate the membrane-binding activity of upstream domains (myr,

PB1-3) which have been well-characterized as necessary for membrane localization.

These results also suggest that activity at this domain may in fact regulate the

redistribution of gravin. While this activity is likely to involve Ca^{2+}/calmodulin binding,

future studies will be required to understand the precise function of Ca^{2+}/calmodulin at

this region.

ATP Mediated Gravin Redistribution Involves Both Ca^{2+} and PKC

Since gravin was demonstrated to undergo redistribution from the cell membrane

to the cytosol after ionomycin and thapsigargin treatment, we sought to determine if

gravin redistribution would occur through receptor signaling linked to $[Ca^{2+}]_i$ elevation.

ATP is well known to induce an increase in $[Ca^{2+}]_i$ through both ionotropic (P2X) and

metabotropic (P2Y) purinergic receptors. While P2X receptors trigger the influx of

extracellular calcium, P2Y receptors stimulate cytosolic calcium increases primarily

through their association with $G_{q/11}$, which activates PLCβ to stimulate both PKC

activation and InsP$_3$ mediated release of calcium from intracellular stores (Erb, Liao,

Seye, & Weisman, 2006; Weisman et al., 2006). Treatment of HEC-1A cells with 10 mM

ATP resulted in a change in gravin-EGFP distribution from the cell cortex to the cytosol.

This change was also accompanied by an immediate increase in $[Ca^{2+}]_i$ followed by a

subsequent decrease back to basal levels (Fig. III-5A,B). ATP-mediated gravin

redistribution and $[Ca^{2+}]_i$ increase also occurred in Ca^{2+} free SES (Fig. III-5C,D), a result

consistent with P2Y receptor activation.

To determine the mechanism of ATP-mediated gravin redistribution in HEC-1A

cells, inhibition studies were performed using bisindolylmaleamide (BIM, 1 μM), a PKC

inhibitor, and/or BAPTA-AM (50 μM). Treatment with 10mM ATP caused complete

redistribution of gravin from the cell membrane to the cytosol, compared to cells with no ATP added (Fig. III-6A-D). ATP treatment in the presence of either BIM or BAPTA-AM (Fig III-6E-H) caused partial redistribution of gravin, while ATP treatment in the presence of both inhibitors showed no gravin redistribution (Fig. III-6I-J). Measurement of the ratio of membrane to cytosolic fluorescence at each time point revealed that BIM and BAPTA-AM significantly attenuated ATP-mediated gravin redistribution (Fig. III-6K,L). However, treatment with both BAPTA-AM and BIM together was necessary to completely prevent gravin redistribution in cells treated with ATP (Fig. III-6M), suggesting that both Ca^{2+} and PKC are required for ATP-mediated gravin dynamics. Statistical analyses were performed at each time point using a Mann-Whitney Rank Sum Test (horizontal bars denote time points at which $p < 0.05$). Analysis of the maximum change in membrane to cytosolic ratio for each of the treatments further revealed three statistically significant subsets: ATP in the presence of no inhibitors showed the greatest change in gravin distribution; ATP in the presence of either BIM or BAPTA-AM showed a significantly reduced change in gravin distribution; and ATP in the presence of both BIM and BAPTA-AM showed no difference in gravin distribution from untreated cells (ANOVA followed by Holm-Sidok post-hoc, significantly different subsets denoted by asterisks, $p < 0.05$) (Fig. III-6N).

Loss of Cortical PKA Compartmentalization Following Gravin Redistribution.

Since a major function of AKAPs is to direct PKA to specific subcellular compartments, we sought to examine whether Ca^{2+}-mediated or purinergic receptor-mediated gravin redistribution also causes the redistribution of PKA. We used confocal microscopy to simultaneously visualize the subcellular distribution of gravin-EYFP and

PKA RII-ECFP before and after treatment with ionomycin, thapsigargin, or ATP. As seen in Fig. III-7, expression of PKA RII-ECFP was distributed throughout the cytosol in cells transfected with PKA RII-ECFP alone or when co-transfected with a gravin construct lacking the PKA-binding domain, (ΔPKA) gravin-EYFP, but PKA RII-ECFP was concentrated at the cell periphery in cells co-expressing WT gravin-EYFP. Treatment of cells with ionomycin, thapsigargin, or ATP resulted in in the loss of PKA RII-ECFP localization at the cell cortex, concurrent with the redistribution of gravin-EYFP (Fig. III-7A-F'). These studies demonstrate that signaling events that trigger translocation of gravin also alter the subcellular distribution of PKA.

Discussion

The aim of the current study was to characterize the redistribution of gravin following an increase in $[Ca^{2+}]_i$ and determine its effect on subcellular PKA localization. This study demonstrated that gravin undergoes subcellular redistribution following treatment with ionomycin or thapsigargin, both from extracellular Ca^{2+} influx and from intracellular store release. Calcium mediated redistribution of gravin does not require the presence of polybasic domains 1-3, three regions rich in basic amino acids which bind Ca^{2+}/calmodulin and are involved in targeting gravin to the plasma membrane. Interestingly, deletion of a fourth calmodulin binding domain (amino acids 670-694) which is reported to not associate with phospholipid vesicles (Tao et al., 2006) resulted in a dramatic decrease in the localization of gravin at the cell cortex. This study also demonstrated that purinergic receptor mediated elevation of $[Ca^{2+}]_i$ and activation of PKC can also induce gravin redistribution. Finally, ionomycin, thapsigargin, and ATP mediated gravin redistribution also triggered the loss of PKA localization at the cell

cortex. Our results support the hypothesis that receptor mediated signaling events involving calcium and/or PKC can influence cAMP-dependent signaling through the spatial regulation of gravin.

Calcium mediated redistribution of gravin was first reported by Tao et al. (Tao et al., 2006) to occur in response to A23187, a calcium ionophore. The current study adds to this finding in several important ways. First, our approach revealed that gravin redistribution occurs immediately following treatment with the calcium elevating agents ionomycin, thapsigargin, or ATP, and that these agents caused the complete redistribution of gravin from the cell periphery to the cytosol in as little as 60 seconds. Second, our findings with ionomycin and thapsigargin demonstrated that gravin redistribution can be mediated by both influx of extracellular calcium and the release of calcium from intracellular stores. Third, our approach demonstrated that both sustained and transient increases in cytosolic calcium following ionomycin or thapsigargin treatment were sufficient to mediate the complete redistribution of gravin. Finally, the current study demonstrated that Ca^{2+} signaling is involved in receptor-mediated gravin redistribution following ATP treatment. In these experiments ATP-generated Ca^{2+} transients were sufficient to cause partial gravin redistribution, in contrast to experiments involving ionomycin and thapsigargin, which caused complete gravin redistribution. This apparent contrast suggests that while Ca^{2+} alone may be sufficient for complete gravin redistribution under certain conditions, the mechanisms involved in receptor-mediated gravin redistribution may be more complex. Indeed, our findings demonstrate that ATP-mediated gravin redistribution also acted through PKC activity in addition to $[Ca^{2+}]_i$ elevation. Previous reports have shown that PKC activation causes gravin redistribution

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and complements our finding that PKC is involved in ATP-mediated gravin redistribution (Nelson, Moissoglu, Vargas, et al., 1999; Piontek & Brandt, 2003; X. Yan et al., 2009). In sum, the results of the current study further our understanding of the real-time dynamics behind Ca^{2+} mediated gravin redistribution, and provide additional insight into its biological context and molecular mechanism.

Although the mechanism behind calcium mediated redistribution of gravin has yet to be fully elucidated, previous work indicates the involvement of Ca^{2+}/calmodulin (CaM). Tao et al. (Tao et al., 2006) reported compelling evidence that CaM interacts with the membrane-binding polybasic domains (PB1-3) of gravin to cause the dissociation of these domains from phospholipid vesicles, but this work used gravin constructs lacking the N-terminal myristoylation sequence. More recently, work in our lab demonstrated that the myristoylation sequence is sufficient to target gravin to the plasma membrane, even in the absence of the polybasic domains (13). We therefore postulated that a myristoylated gravin construct lacking the polybasic domains would not undergo redistribution in response to elevated $[Ca^{2+}]$. Surprisingly, such a construct underwent redistribution from the plasma membrane to the cytosol at the same rate as full-length gravin. This finding indicates that CaM binding to the polybasic domains does not fully explain the mechanism for Ca^{2+} mediated redistribution of myristoylated gravin. Indeed, the membrane-binding activity of the myristoylation site must also be altered by $[Ca^{2+}]_i$ elevation. Our results suggest that CaM interaction with an additional calmodulin binding domain, termed CB4, may regulate the membrane targeting of gravin. This CB4 domain conforms to the 1-5-10 consensus sequence for CaM binding ([669]KRK$\underline{V}$DTS$\underline{V}$SWEA$\underline{L}$ICV) and is identical to the SSeCKS-3 domain found in the rat

orthologue of gravin, reported by Lin and Gelman to bind CaM *in vitro* (Lin & Gelman, 2002). Deletion of the four known gravin calmodulin binding domains (ΔPB1-3, CB4) resulted in a dramatic loss of membrane localization of gravin, while reconstitution of only the CB4 domain fully restored membrane localization to that of full-length gravin. Moreover, deletion of CB4 alone caused a similar loss of membrane localization to that of (ΔPB1-3,CB4) gravin-EGFP. These results are the first to demonstrate that the CB4 domain is critical in the subcellular localization of gravin. Although the precise effect of CB4 deletion is unclear, one possibility is that CB4 deletion mimics CaM binding to gravin. Nonetheless, our results support the notion that calcium-mediated gravin redistribution operates through a mechanism which involves the interaction of CaM with gravin.

Our findings that gravin redistribution occurs from both intracellular calcium influx and from the release of calcium from intracellular stores suggest that gravin redistribution occurs across a variety of physiological contexts. These modes of calcium signaling are central to cellular homeostasis and exist not only in G protein coupled receptor (GPCR) systems, but also in the form of plasma membrane channels such as ionotropic receptors, cyclic-nucleotide gated channels, L-type channels, and membrane components of store operated calcium entry (SOCE). Thus, signaling through any one of a number of pathways that induce an increase in cytosolic $[Ca^{2+}]$ may result in the redistribution of gravin and affect the signaling dynamics of molecules associated with gravin. Our finding that ATP triggered redistribution of gravin through a pathway that involved both $[Ca^{2+}]_i$ increase and PKC activation supports this notion and indicates that GPCR signaling through the canonical $G_{q/11}$ pathway may be a major pathway for gravin

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redistribution. Given the widespread occurrence of signaling pathways involving these modes of signaling, gravin redistribution is likely to be a widespread response to signaling events and could serve as an important mediator for cross-talk over the signaling dynamics of molecules bound to the gravin scaffold. Since gravin interacts with a diverse array of signaling molecules including PKA, PKC, PDE4, β2-adrenergic receptor (β2AR), cyclin D and others (Gelman, 2002; Gelman & Gao, 2006; Tao et al., 2003; Willoughby et al., 2006a), subcellular translocation of this AKAP would likely affect signaling events involving these binding partners. PKA, for instance, is known to require spatial compartmentalization by AKAPs (Skroblin, Grossmann, Schafer, Rosenthal, & Klussmann, 2010). Thus, loss of cortically-localized gravin/PKA would likely affect PKA signaling by reducing activity at the plasma membrane or directing PKA signaling to another subcellular compartment. β2AR signaling is known to be regulated by gravin expression and redistribution in a variety of contexts (G. Fan et al., 2001; F. Lin, H. Wang, et al., 2000; Shih et al., 1999a; Tao et al., 2006; Tao et al., 2003) and thus receptor mediated events leading to gravin redistribution would most certainly impact a wide range of β2AR dependent physiological activities known to be linked to gravin. Finally, reports that PDE4 binds to gravin and that this complex regulates cortical cAMP levels suggest that receptor mediated relocalization of gravin could impact cAMP dependent signaling broadly by altering dynamic control of [cAMP].

Conclusions

We report that gravin undergoes rapid redistribution from the cell periphery to the cytosol following treatment with the calcium-elevating agents ionomycin, thapsigargin, or ATP, and that this redistribution is accompanied by a change in subcellular PKA

localization. The effect of ionomycin and thapsigargin on gravin distribution was calcium dependent, whereas ATP's effect on gravin distribution involved both calcium and PKC. Surprisingly, calcium mediated redistribution of myristoylated gravin did not require polybasic regions 1-3, but deletion of a 1-5-10 consensus sequence for calmodulin binding downstream of the polybasic regions seems to regulate the targeting of gravin to the cell cortex. Our data supports the hypothesis that receptor mediated signaling events involving calcium and/or PKC can alter cAMP-dependent signaling through the spatial regulation of gravin and anchored PKA. This finding suggests that gravin facilitates a novel cross-talk mechanism in which cAMP-dependent signaling pathways are altered by calcium and PKC, and lays the groundwork for future studies of gravin spatiotemporal dynamics in regulating cAMP-dependent signaling events.

Acknowledgements
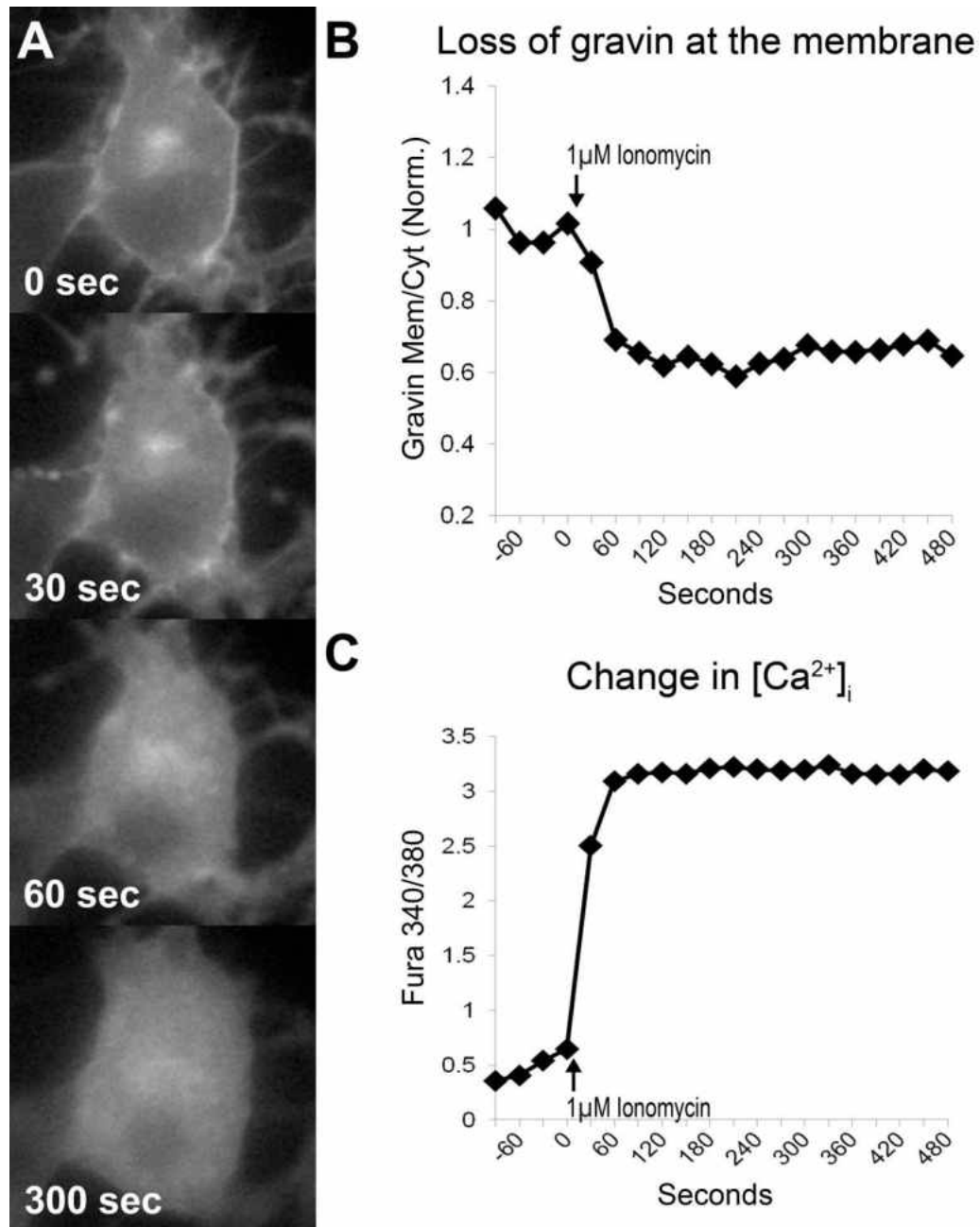
Figure III-1. Ionomycin treatment induces gravin redistribution. (A) Fluorescent micrographs illustrating that gravin-EGFP undergoes redistribution immediately following the addition of 1 µM ionomycin. (B) Plot of membrane:cytosol fluorescence intensity ratio over time illustrating the time course of gravin redistribution. (C) Corresponding plot of the Fura2 fluorescence ratio illustrating the rapid increase in $[Ca^{2+}]_i$ upon ionomycin treatment. Note that gravin translocation is complete at t = 60 seconds, which corresponds to the time at which maximal cytosolic calcium increase is observed.
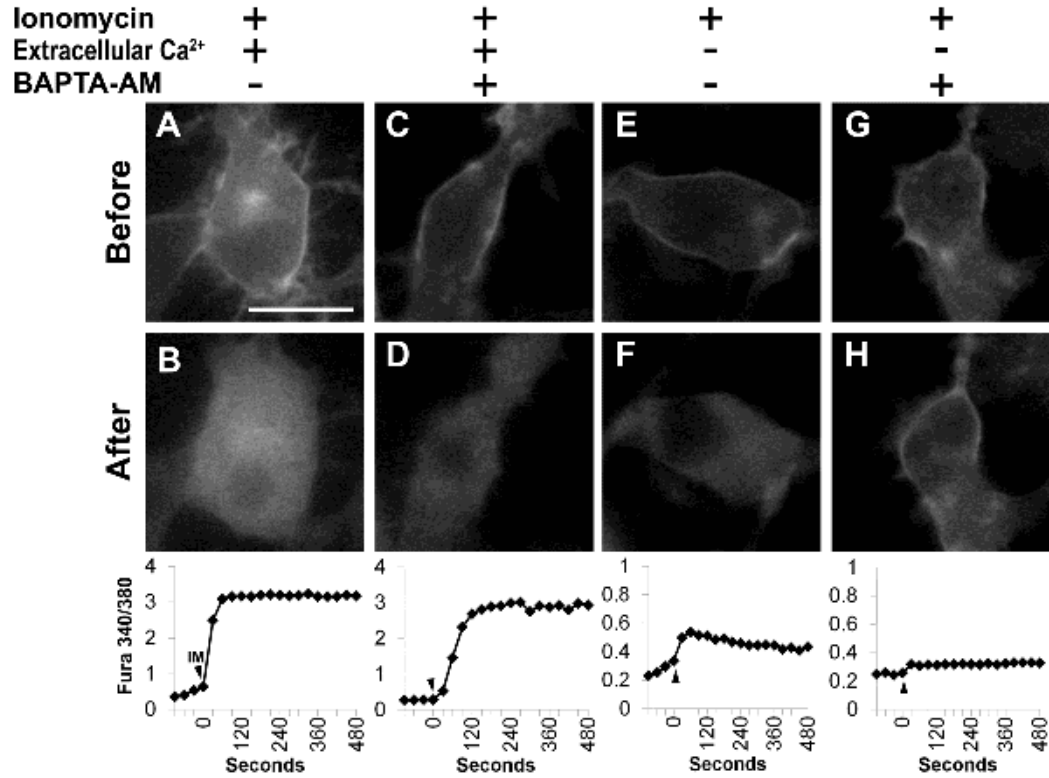
Figure III-2.   Representative fluorescent images of gravin-EGFP and corresponding
plots of Fura2 fluorescence ratios illustrate that ionomycin-mediated
gravin distribution is calcium dependent. Note that ionomycin treatment in
both the presence and absence of extracellular calcium resulted in
elevation of intracellular calcium and redistribution of gravin. Ionomycin
treatment in the presence of BAPTA-AM (10 µM) and extracellular
calcium also resulted in a sustained increase in cytosolic calcium and
gravin redistribution. However, when BAPTA-AM was present in Ca^{2+}
free medium, ionomycin-mediated gravin redistribution was fully
prevented. Scale bar = 10 µM.
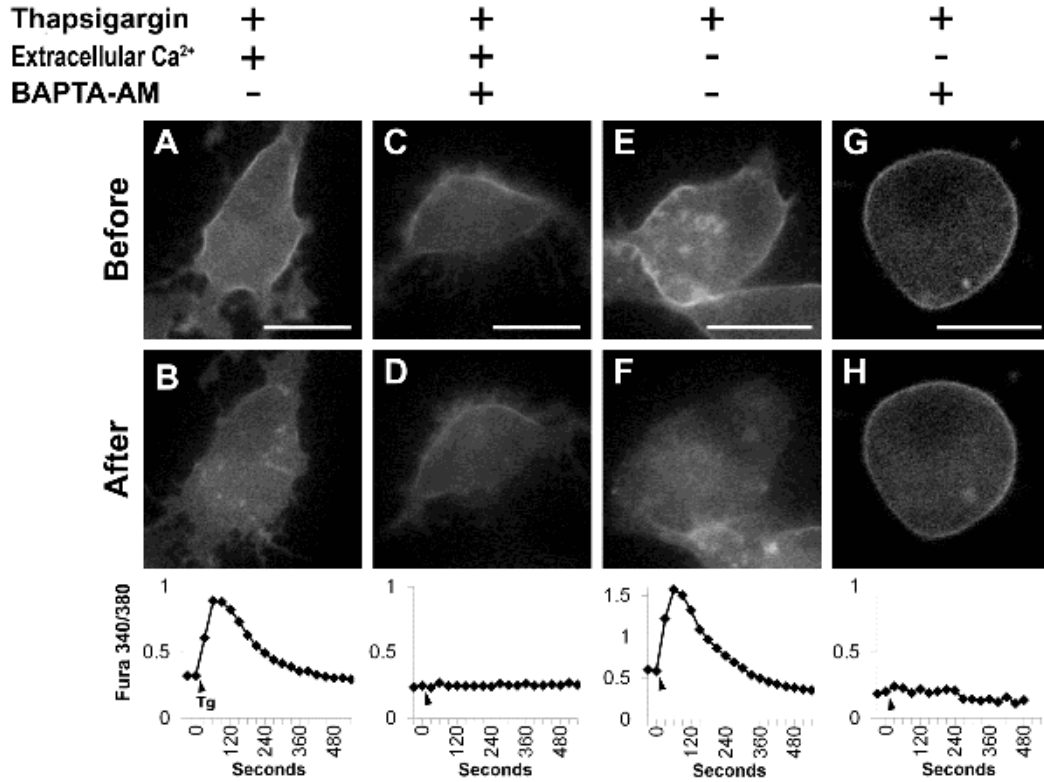
Figure III-3.   Representative fluorescent images of EGFP-gravin and corresponding plots of Fura2 fluorescence ratios illustrating that thapsigargin mediated gravin redistribution is calcium dependent. Note that thapsigargin treatment in the presence and absence of extracellular calcium caused redistribution of gravin concurrent with a transient increase in cytosolic calcium. Addition of BAPTA-AM (10 μM) prior to thapsigargin treatment fully prevented gravin redistribution in either the presence or absence of extracellular Ca^{2+}. Scale bar = 10 μM.

Figure III-4. Role of four calmodulin binding domains (PB1-3, CB4) in gravin redistribution and subcellular localization. (A, B) Comparison of the rate of redistribution from cell membrane to cytosol for either WT gravin-EGFP and (ΔPB1-3) gravin-EGFP (A) or WT gravin-EGFP and (ΔPB1-3+) gravin-EGFP (B) after ionomycin treatment. The deleted regions are illustrated below the graphs. No difference was observed between WT gravin and (ΔPB1-3) gravin, but the rate of (ΔPB1-3+) gravin translocation from membrane to cytosol was significantly reduced compared to WT gravin. (Comparison at each time point revealed significant differences between constructs as indicated by the horizontal bar; Mann-Whitney Rank Sum Test, $p < 0.05$). (C) Graph illustrating the effect of deleting the CB4 region on localization of gravin at the cell periphery in transfected cells. Note that localization of the (ΔPB1-3, CB4) mutant at the cell cortex was significantly reduced compared to WT gravin. Reconstitution of the CB4 domain (ΔPB1-3+) restored membrane localization, while deletion of the CB4 domain (ΔCB4) alone resulted in a decrease in membrane localization similar to that of ΔPB1-3,CB4. Astericks indicate significant differences from WT gravin-EGFP (one-way ANOVA followed by a Holm-Sidak post-hoc test; $p < 0.05$). (D) A Western blot demonstrating the expression of full-length gravin–EGFP and its deletion mutants in AN3CA cells. Sixty micrograms total protein was loaded in all lanes. The number of amino acids comprising each construct is marked at the top of the blot.
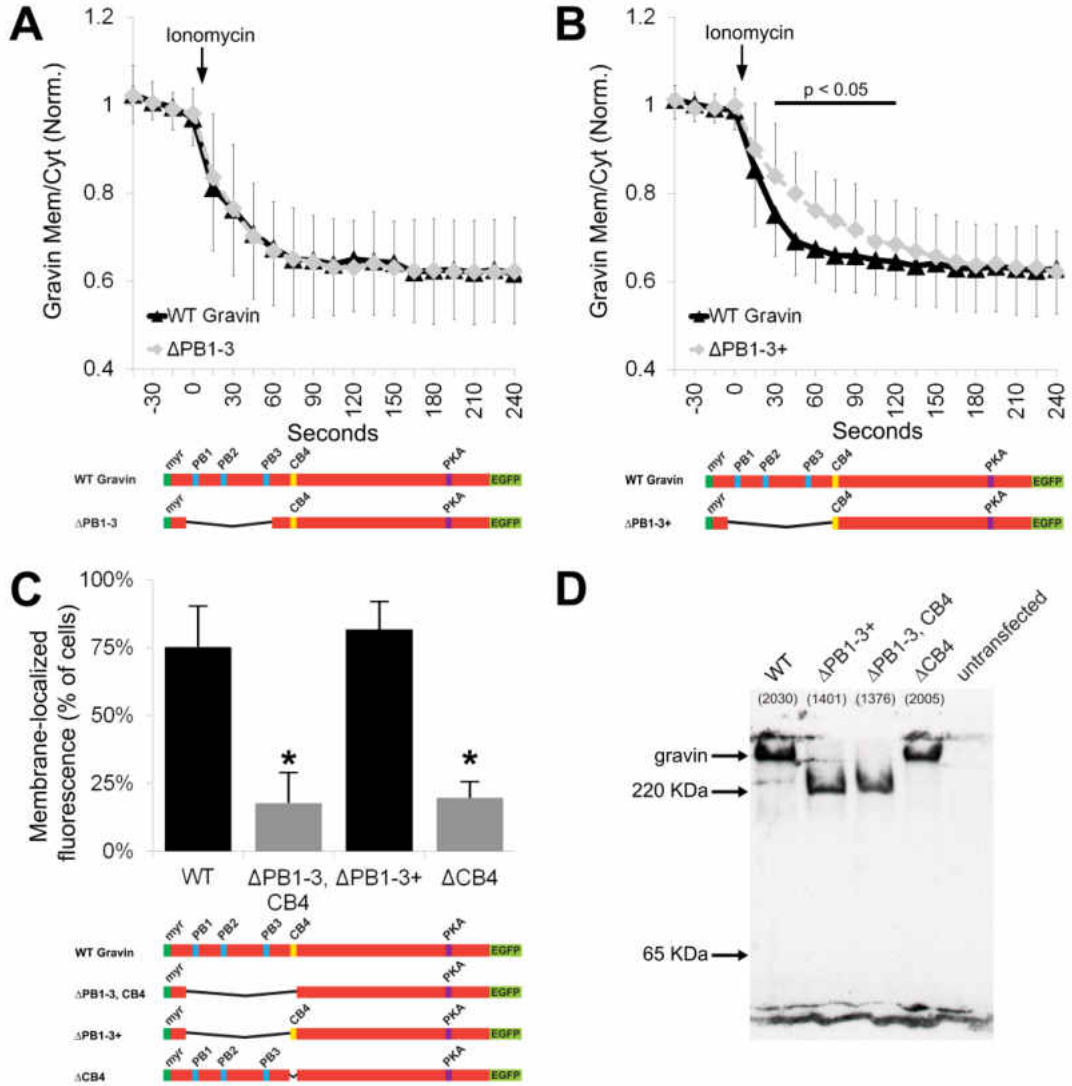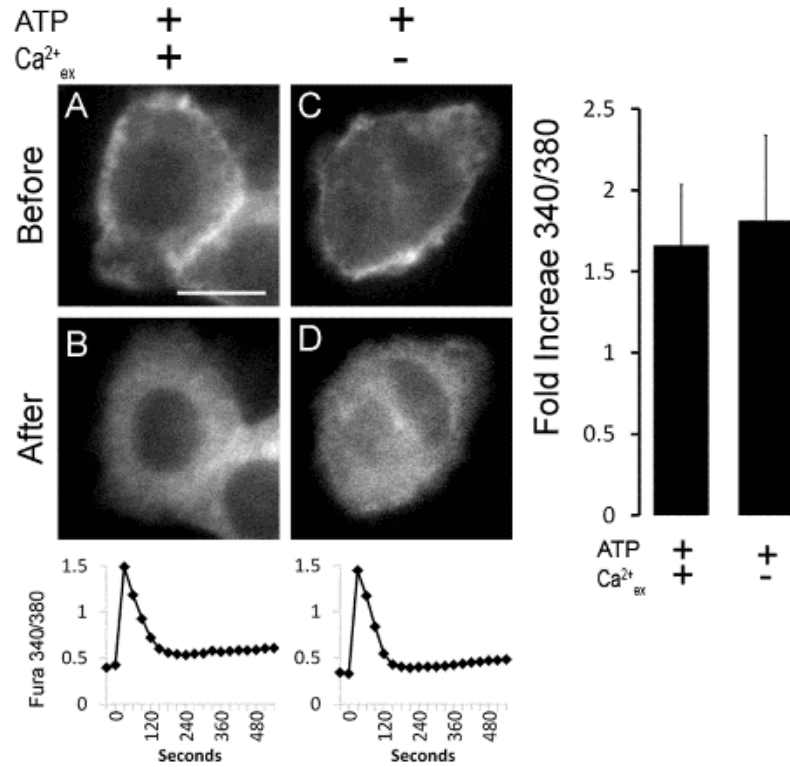
Figure III-5. Fluorescence micrographs illustrating the effect of ATP treatment on gravin redistribution and changes in intracellular calcium in the presence and absence of extracellular calcium. Gravin redistribution was observed following ATP treatment (10 mM) in both regular SES and Ca^{2+} free SES. Moreover, cytosolic $[Ca^{2+}]$ increased to the same extent (an approximately 1.7 fold increase) in cells treated with ATP in regular and Ca^{2+} free SES. Scale bar = 10 μM.

Figure III-6.   Regulation of gravin redistribution by ATP treatment. (A-J) Fluorescence micrographs illustrating the effect of ATP treatment (10 mM) on gravin distribution in the presence of bisindolylmaleimide (BIM), BAPTA-AM, or BIM and BAPTA-AM together. ATP treatment induced complete redistribution of gravin (C,D) compared to untreated cells (A,B). Gravin redistribution was partially attenuated when ATP was administered in the presence of 2 μM bisindolylmaleimide (BIM) or 50 μM BAPTA-AM (G,H). However, gravin redistribution was fully prevented when ATP was administered in the presence of both BIM and BAPTA-AM (I,J). (K-M) Quantitative analysis of gravin-EGFP dynamics in response to ATP in the presence of BIM and BAPTA-AM. BIM (K) or BAPTA-AM (L) caused a significant attenuation in the redistribution of gravin-EGFP. However, ATP treatment in the presence of both BIM and BAPTA-AM completely inhibited redistribution of gravin-EGFP (M). (N) Histogram illustrating the extent to which gravin distribution changed under the different treatment conditions. The height of the bars corresponds to the difference between the membrane/cytosol ratio at t=0 and the minimum membrane/cytosol ratio reached during each run. One-way ANOVA followed by Holm-Sidak post hoc tests revealed three significantly distinct responses to the treatments, which are denoted by asterisks ($p < 0.05$). Scale bar = 10 μM.
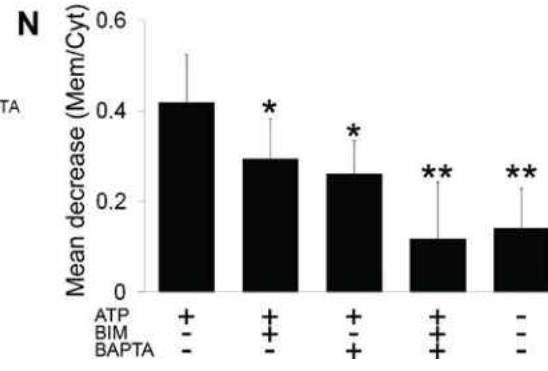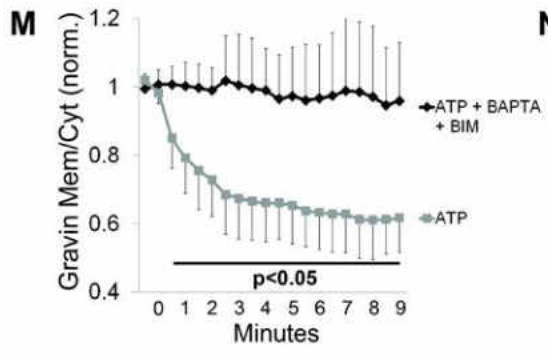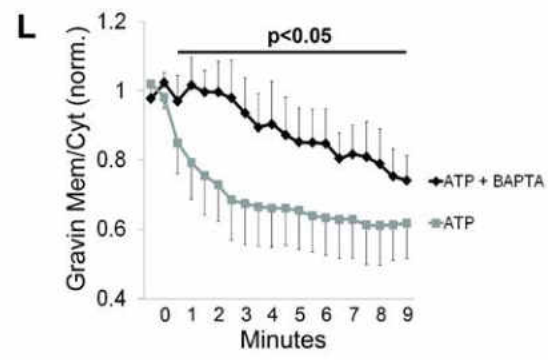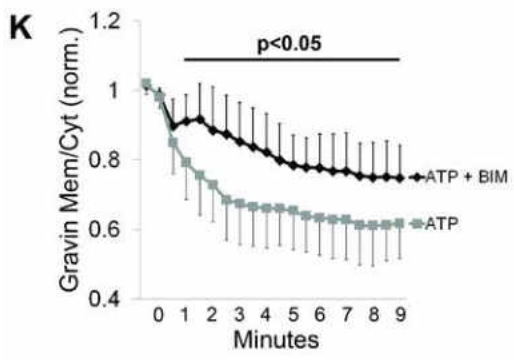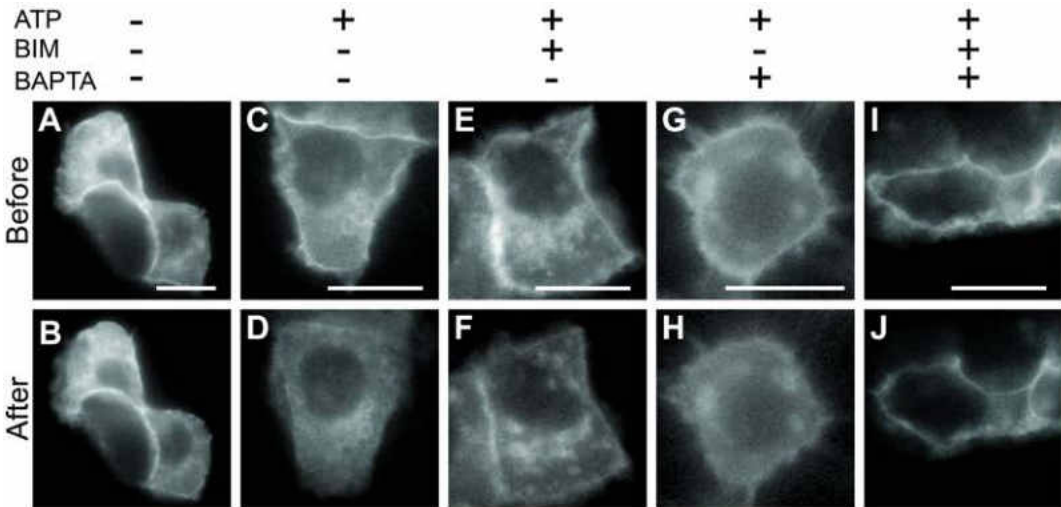
Figure III-7.   Confocal micrographs of HEC1A cells transfected with gravin-EYFP and PKA RII-ECFP. PKA co-distributes with gravin at the cell cortex in cells expressing both PKA and full-length gravin (A,A'; C,C', E,E'), However, redistribution of gravin following treatment with ionomycin (B, 1 µM), thapsigargin (D, 0.4 µM), or ATP (F, 10mM) triggers the loss of PKA localization at the cell periphery (B', D', F') in concert with redistribution of gravin. PKA RII-ECFP did not localize at the cell cortex nor change distribution after ionomycin treatment in cells co-expressing PKA RII-ECFP and a gravin–EYFP construct lacking the PKA RII binding domain (Δ-PKA gravin; G,G'; H,H'), although the gravin construct underwent redistribution to the cytosol. Controls transfected with either gravin-EYFP or PKA RII-ECFP alone confirmed that the observed codistribution of gravin and PKA was not due to cross-over of the EYFP signal into the ECFP channel, or vice versa. Scale bar = 10 µM.

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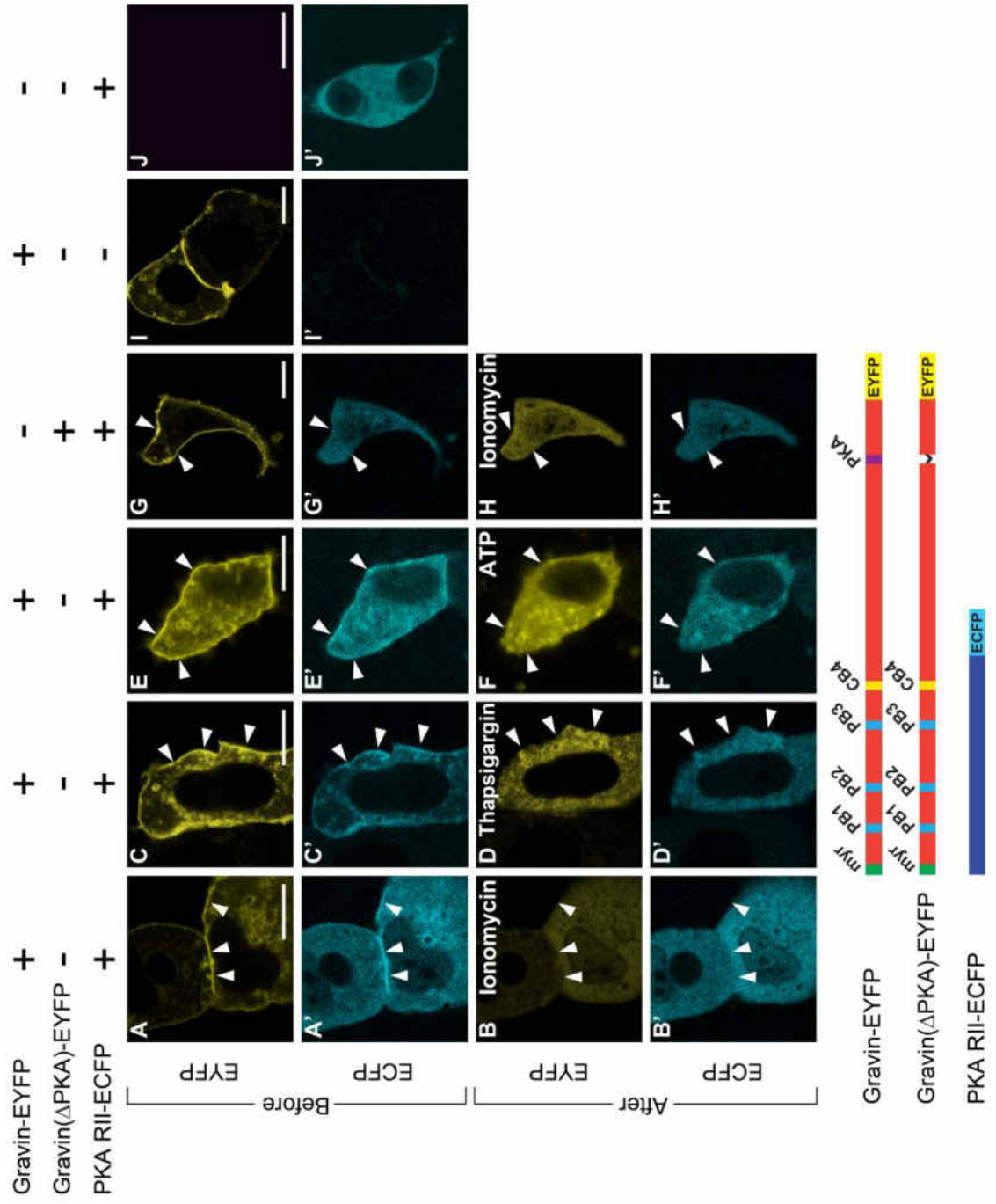
CHAPTER IV

FRET BIOSENSORS REVEAL AKAP-MEDIATED SHAPING OF SUBCELLULAR
PKA ACTIVITY AND A NOVEL MODE OF Ca^{2+}/PKA CROSSTALK

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Abstract

Scaffold proteins play a critical role in cellular homeostasis by positioning signaling molecules in proximity to downstream effectors. However, some scaffold proteins do not assemble static enzyme complexes, but form dynamic signalosomes that traffic to different subcellular compartments in response to stimuli. Gravin (AKAP12), a multivalent scaffold linked to a variety of cellular functions, anchors PKA and other enzymes to the plasma membrane but is redistributed to the cytosol upon elevation of $[Ca^{2+}]_i$. We postulate that gravin redistribution represents a novel mechanism for crosstalk between Ca^{2+}-dependent and cAMP-dependent signaling pathways. To assess this, we measured the impact of gravin-V5/His expression on compartmentalized PKA activity using the PKA FRET biosensor AKAR3. Expression of gravin-V5/His in AN3 CA cells, which lack endogenous gravin, caused an increase in forskolin-stimulated PKA activity in AKAR3 constructs targeted to the plasma membrane compared to control cells lacking gravin or expressing a gravin construct lacking the PKA-binding domain. Expression of a gravin mutant with reduced membrane localization also showed no increase in membrane PKA activity compared to control cells lacking gravin. In addition, gravin-V5/His caused a decrease in forskolin-stimulated PKA activity in cytosolic AKAR3 constructs compared to control cells lacking gravin, and this effect also required PKA interaction and membrane localization. Pretreatment with thapsigargin, a calcium elevating agent, stimulated gravin redistribution away from the membrane and prevented gravin's impact on membrane PKA activity. These results reveal that gravin shapes the subcellular profile of PKA activity and support the hypothesis that gravin mediates crosstalk between Ca^{2+} and cAMP-dependent signaling pathways. Based on these results,

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AKAP localization dynamics may represent an important paradigm for the regulation of cellular signaling networks.

Introduction

Intracellular signal transduction requires precise physical interactions between specific signaling proteins within a receptor-directed cascade. It is now clear that many of these protein-protein interactions are facilitated by scaffold proteins and not by random diffusion (Good et al., 2011). A-Kinase Anchoring Proteins (AKAPs) play an integral role in this by compartmentalizing cAMP-dependent protein kinase (PKA) and other enzymes to specific subcellular locations. AKAPs possess a conserved amphipathic helical domain that binds the regulatory subunit of cyclic AMP-dependent kinase (PKA) and a subcellular targeting domain that serves to anchor PKA and often additional kinases, phosphatases, and other regulatory enzymes to diverse subcellular compartments (reviewed in Wong & Scott, 2004). Interestingly, some AKAPs do not merely serve as static "anchors", but can traffic to alternative subcellular compartments in response to stimuli (Eide et al., 1998; H. Li, Adamik, Pacheco-Rodriguez, Moss, & Vaughan, 2003; Rawe, Payne, Navara, & Schatten, 2004; K. E. Smith, Gibson, & Dell'Acqua, 2006).

Gravin (AKAP12) is a 300 kD AKAP with dramatic spatial targeting dynamics. Gravin binds to PKA and a host of other signaling enzymes and associates with the plasma membrane through an N-myristoylation site and three polybasic domains (PB1-3) which are presumed to adhere electrostatically to acidic phospholipids. In response to either PKC activation or intracellular calcium ($[Ca^{2+}]_i$) elevation, gravin is redistributed away from the membrane, removing gravin bound PKA from the cell periphery. Yan et al (X. Yan et al., 2009) showed that PKC activation triggers the redistribution of

gravin/PKA to a juxtanuclear vesicular compartment. In addition, Tao et al. (Tao et al., 2006) were the first to show that elevation of intracellular $[Ca^{2+}]$ triggers the redistribution of gravin to the cytosol through a mechanism thought to involve Ca^{2+}/calmodulin binding to PB1-3. A recent study from our laboratory further revealed that calcium mediated gravin redistribution triggers relocalization of PKA away from the membrane and that a fourth putative calmodulin binding domain called CB4 may be critical in this event. In addition, this study found that receptor-mediated signaling triggers gravin/PKA redistribution to the cytosol through a mechanism involving both calcium and PKC (Schott & Grove, 2013). These findings raise the interesting possibility that gravin facilitates a crosstalk mechanism in which Ca^{2+}/PKC-dependent inputs can modulate cAMP-dependent outputs through the redistribution of gravin. However, gravin's impact on subcellular PKA activity under both basal conditions and upon Ca^{2+}/PKC-mediated redistribution is currently unknown. This could have important implications for gravin's role in many health-and disease-related contexts that utilize crosstalk between Ca^{2+}/PKC-dependent and cAMP-dependent signaling, such as cellular migration (Akakura & Gelman, 2012; Gelman et al., 2000; Liu, Guan, Hu, Gu, & Lu, 2011), cancer (reviewed in Gelman, 2012), learning and memory (Havekes et al., 2012), cardiac function (Guillory et al., 2013), and vascular biology (Grove & Bruchey, 2001; Weissmuller et al., 2014).

In the current study, we investigate the role of gravin in shaping subcellular PKA activity and in mediating crosstalk between Ca^{2+} and cAMP-dependent signaling pathways. Gravin's role in targeting PKA to the plasma membrane suggests that gravin potentiates PKA signaling at the plasma membrane. This suggestion in turn implies that

Ca^{2+} elevation may suppress plasma membrane PKA signaling by triggering the redistribution of gravin/PKA into the cytosol. We hypothesize that through this mechanism, gravin mediates cross-talk between calcium and cAMP-dependent signaling pathways. We tested this hypothesis in AN3 CA cells, a cell line that lacks endogenous gravin, by using the FRET-based PKA biosensor AKAR3 that has been optimized to measure PKA activity within targeted subcellular compartments (Allen & Zhang, 2006). Using AKAR3 constructs targeted to the plasma membrane and to the cytosol, we measured the impact of exogenous gravin-V5/His expression on compartmentalized PKA activity at the plasma membrane and in the cytosol. In addition, we tested the impact of calcium-mediated gravin redistribution on plasma membrane PKA activity.

<div align="center">Results</div>

<div align="center">*Forskolin Treatment Stimulates AKAR3 Dynamics*</div>

Fluorescent micrographs in Figure IV-1 illustrate the subcellular distributions of AKAR3 constructs used in this study. AKAR3-NES contains a nuclear export signal which confines it to the cytosol, while AKAR3-CAAX and Lck-AKAR3 are localized to the plasma membrane. After background subtraction, FRET and CFP intensity values over time were measured for each cell within regions of interest using ImageJ. FRET intensity values were then corrected for CFP crossover (44% based on images of cells expressing CFP only), and ratios of FRET/CFP intensities were plotted over time for each cell measured. Upon treatment with forskolin, fluorescence intensity in the CFP channel decreased while FRET intensity increased (Fig. IV-1A), causing an increase in the ratio of FRET/CFP emissions which peaked close to 60 seconds (Fig. IV-1B). FRET/CFP ratios were normalized by dividing each ratio in the time series by the mean of ratio

values prior to treatment. To calculate the fold increase in FRET/CFP as a result of forskolin treatment, the max ratio value for each cell, R_{max}, was divided by the mean ratio value prior to treatment, R_0 (Fig. IV-1B-C).

Role of Gravin in Shaping Subcellular PKA Activity

To determine the effect of gravin-V5/His expression on subcellular PKA activity, three different AKAR3 constructs were used which were localized to the plasma membrane (AKAR3-CAAX and Lck-AKAR3) and in the cytosol (AKAR3-NES). AKAR3-CAAX contains a motif reported to target non-raft plasma membrane microdomains, whereas Lck-AKAR3 contains a motif reported to target membrane rafts (Agarwal et al., 2014; Allen & Zhang, 2006; Depry, Allen, & Zhang, 2011; Gao et al., 2011; Melkonian, Ostermeyer, Chen, Roth, & Brown, 1999; Zacharias, Violin, Newton, & Tsien, 2002). AKAR3-NES contains a nuclear export signal which restricts this biosensor to the cytosol (Allen & Zhang, 2006). The AN3 CA cells used in this study do not express gravin endogenously (Schott & Grove, 2013; X. Yan et al., 2009). Prior to microscopy, cells were equilibrated for 30 minutes in standard extracellular solution (SES) containing the phosphodiesterase inhibitor IBMX (10 μM) to prevent cAMP degradation. To stimulate cAMP elevation and subsequent PKA activity, we treated cells with the adenylyl cyclase activator forskolin (10 μM) and measured changes in AKAR3 fluorescence in the presence and absence of gravin-V5/His. Upon treatment with forskolin, we observed a sustained increase in the ratio of AKAR3 FRET/CFP fluorescence which reached its peak value by 60 seconds. As seen in Fig. IV-2A-B, gravin-V5/His expression significantly elevated the response of AKAR3-CAAX from 1.112 to 1.205, an increase of 45%. Gravin-V5/His expression also elevated the response

of Lck-AKAR3 from 1.045 to 1.072, an increase of 37% (Fig. IV-2C-D). In the cytosol, gravin-V5/His expression suppressed the response of AKAR3-NES from 1.223 to 1.178, a decrease of 26% (Fig. IV-2E-F). From these results, we conclude that gravin expression potentiates PKA activity in the plasma membrane in a manner which may be slightly greater in non-raft membrane domains vs membrane rafts, but reduces PKA activity in the cytoplasm. It is likely that gravin expression accomplishes this by anchoring PKA to the plasma membrane and sequestering PKA away from the cytosol.

Gravin-PKA Interaction is Required for Gravin-Mediated
Shaping of Subcellular PKA Activity
.

We next sought to examine the role of gravin-PKA interaction in gravin-mediated shaping of subcellular PKA activity using a gravin construct lacking its PKA-binding domain. First, we quantified the subcellular localization of PKA RII at the cell periphery in AN3 CA cells co-expressing either full-length (WT) gravin-V5/His or a (ΔPKA) gravin-V5/His mutant which lacks the PKA binding domain. Immunofluorescence labeling of gravin in these cells revealed that roughly 95% of cells transfected with PKA RII-ECFP also expressed gravin-V5/His constructs, and cells expressing PKA RII-ECFP only had no gravin (Fig. IV-3A). As seen in Fig. IV-3B, roughly 40% of cells co-expressing WT gravin-V5/His and PKA RII-ECFP showed ECFP localization along the cell periphery. In contrast, no ECFP localization at the cell periphery was seen in cells co-expressing (ΔPKA) gravin-V5/His and PKA RII-ECFP or in cells expressing PKA RII-ECFP only. These results show that gravin targets PKA RII to the cell periphery, and that the (ΔPKA) gravin-V5/His mutant has no impact on subcellular PKA localization at the cell periphery.

To determine if the gravin-mediated changes in subcellular PKA activity were dependent on gravin-PKA interaction, we co-transfected AN3 CA cells with the AKAR3 constructs and either WT gravin-V5/His or a (ΔPKA) gravin-V5/His. In figure IV-4, confocal micrographs of transfected AN3 CA cells labeled with a gravin antibody and CY3 secondary antibody show that both full-length (WT) gravin-V5/His and (ΔPKA) gravin-V5/His were localized to the plasma membrane. In cells expressing plasma membrane AKAR3-CAAX, gravin-V5/His expression potentiated forskolin-stimulated PKA activity in comparison to control cells with no gravin, but this potentiation was not observed in cells expressing (ΔPKA) gravin-V5/His. Similarly, in cells expressing cytosolic AKAR3-NES, gravin-V5/His expression reduced forskolin-stimulated PKA activity compared to control cells with no gravin, but this reduction was not observed in cells expressing (ΔPKA) gravin-V5/His. These results confirm that gravin-PKA interaction is required for gravin-mediated potentiation of plasma membrane PKA activity and reduction of forskolin-stimulated PKA activity in the cytosol.

Mutation of Gravin's CB4 Domain Reduces Membrane Localization
and Gravin-Mediated Changes in Subcellular PKA Activity.

Gravin localization at the plasma membrane is aided in part by an N-myristoylation sequence and three N-terminal polybasic domains, PB1-3 (X. Yan et al., 2009). Tao et al. (2006) proposed that Ca^{2+}/calmodulin (CaM) binding to PB1-3 is responsible for calcium mediated relocalization of gravin into the cytosol (Tao et al., 2006). However, more recently we reported that deletion of PB1-3 had no effect on the ability of calcium to cause this relocalization. Moreover, this previous study found that even in the presence of the myristoylation site and PB1-3, gravin localization at the cell periphery was severely hindered by the deletion of a fourth putative calmodulin binding

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domain (CB4) (Schott & Grove, 2013), a region which contains a 1-5-10 consensus motif for CaM interaction (Rhoads & Friedberg, 1997). To test the role of this CaM consensus sequence within the CB4 domain in the membrane localization of gravin, we used site-directed mutagenesis to substitute the 1-5-10 amino acids in this sequence for alanine as seen in Fig. IV-5A, similar to the strategy used by several other investigators (see Ahn, Lim, Cook, & McDonald, 2004; Fancy et al., 2014; Suever, Chen, McDonald, & Song, 2008). Expressing this mutant construct, termed (mutCB4) gravin-EGFP, revealed a dramatically reduced membrane localization compared to WT gravin-EGFP. Membrane localization of (mutCB4) gravin-EGFP was not different compared to (ΔCB4) gravin-EGFP (Fig. IV-5B). From these results, we conclude that the 1-5-10 amino acids within gravin's CB4 domain are required for normal membrane localization. This suggests a novel role for CaM in supporting gravin localization at the membrane, which contrasts with previous reports showing that CaM promotes gravin relocalization away from the membrane by binding to PB1-3.

Using the (mutCB4) gravin-V5/His construct, we sought to define the importance of gravin localization at the membrane in gravin's ability to impact subcellular PKA activity both at the plasma membrane and within the cytosol. In figure IV-6, representative confocal micrographs of AN3 CA cells transfected with gravin-V5/His constructs and immunolabeled for gravin illustrate that while full-length (WT) gravin strongly localized along the cell periphery, (mutCB4) gravin did not. In AN3 CA cells expressing plasma membrane AKAR3-CAAX, gravin-V5/His expression potentiated forskolin-stimulated PKA activity compared to control cells with no gravin, but this increase was not observed in cells expressing (mutCB4) gravin-V5/His (Fig. IV-6A-B).

Similarly, in cells expressing cytosolic AKAR3-NES, the presence of gravin-V5/His reduced forskolin-stimulated PKA activity in the cytosol compared to control cells with no gravin, but this suppression was not observed in cells expressing (mutCB4) gravin-V5/His (Fig. IV-6C-D). These results demonstrate that alterations in gravin localization have profound effects on subcellular PKA activity, both at the plasma membrane and in the cytosol.

Thapsigargin Causes Gravin Redistribution and Inhibits
Gravin-Mediated Potentiation of Plasma Membrane PKA Activity.

Intracellular calcium elevation causes the redistribution of gravin away from the plasma membrane and into the cytosol, and this redistribution also triggers the relocalization of PKA away from the cell periphery (Schott & Grove, 2013; Tao et al., 2006). In light of this, we sought to determine whether calcium-mediated gravin redistribution would impact the ability of gravin to potentiate PKA activity at the plasma membrane. To test this, we pre-treated AN3 CA cells expressing AKAR3-CAAX with 10 µM thapsigargin, a calcium-elevating agent which causes gravin redistribution. AN3 CA cells co-expressing gravin-V5/His showed elevated PKA activity compared to control cells with no gravin when treated with forskolin without pretreatment with thapsigargin. However, 30 minute pre-treatment with thapsigargin abolished gravin's ability to potentiate forskolin-stimulated PKA activity. Pre-treatment with thapsigargin in cells with no gravin showed no significant difference in forskolin-stimulated PKA activity, confirming that the effect of thapsigargin on plasma membrane PKA activity was gravin-dependent (Fig. IV-7A-B). In Figure IV-7C, representative micrographs of AN3 CA cells expressing gravin-EGFP demonstrate the impact of thapsigargin pretreatment on gravin distribution. To confirm that gravin redistribution had also occurred in experiments

represented by Figure IV-7A-B, coverslips were immunofluorescently labeled for gravin after these experiments. As seen in Figure IV-7D, the percentage of cells showing gravin localization at the cell periphery dropped dramatically with thapsigargin pretreatment. Overall these results demonstrate that calcium-mediated gravin redistribution alters plasma membrane PKA activity and supports the hypothesis that gravin can mediate crosstalk between calcium and cAMP-dependent signaling pathways.

Discussion

In the current study, we investigated the role of gravin in shaping compartmentalized PKA activity and in mediating crosstalk between Ca^{2+} and cAMP-dependent signaling pathways. We report that the expression of gravin potentiated PKA activity at the plasma membrane and reduced PKA activity in the cytosol. The impact of gravin on membrane and cytosolic PKA activity depended on both gravin-PKA interaction and gravin-membrane interaction. This was evident from the finding that mutant gravin constructs that lack PKA interaction ([ΔPKA] gravin) or normal membrane localization ([mutCB4] gravin) had no impact on plasma membrane or cytosolic PKA activity. In addition, redistribution of gravin away from the cell periphery with thapsigargin, a calcium-elevating agent, also inhibited the potentiation of plasma membrane PKA activity by gravin. These results support the hypothesis that gravin mediates cross-talk between calcium and cAMP-dependent signaling pathways and may provide mechanistic insight into gravin's role in health and disease.

In the current study, gravin increased forskolin-stimulated PKA activity at the plasma membrane while reducing cytosolic PKA activity, and this effect required both PKA interaction and localization at the cell periphery. This suggests that gravin mediates

its impact on subcellular PKA activity by targeting PKA to the plasma membrane and sequestering PKA from the cytosol. The notion that gravin targets PKA to the cell periphery is in line with its characterization as an AKAP and has been clearly demonstrated in previous studies (Schott & Grove, 2013; X. Yan et al., 2009). However, the putative sequestration of PKA away from the cytosol is not well defined. Assuming a fixed concentration of PKA, it is logical that AKAP-mediated targeting of PKA to one compartment would to some degree subtract PKA concentrations in other compartments. In fact, this sequestration phenomenon was proposed in an early study of AKAP79 overexpression in HEK293 cells (Ndubuka, Li, & Rubin, 1993). A recent study by Su et al. (2013) also showed that rodent gravin (also called SSeCKS) reduces FAK phosphorylation presumably through the sequestration of Src to lipid rafts and away from FAK-associated complexes (Su et al., 2013). Gravin interaction with cyclin D1 was also shown to sequester cyclin D1 away from the nucleus in mouse glomerular parietal epithelial cells (Burnworth et al., 2012). The current study raises the intriguing possibility that gravin may reduce PKA activity in other cellular compartments through PKA sequestration at the plasma membrane. However, future experiments using endogenous gravin knockdown and/or computational modeling will be required to validate the efficacy of this phenomenon at normal physiologic levels of gravin expression.

The finding that forskolin-stimulated PKA phosphorylation of AKAR3 constructs targeted to the plasma membrane was elevated with the expression of gravin-V5/His suggests that gravin may facilitate PKA phosphorylation of a wide range of membrane-targeted substrates. Although remarkably little is known about gravin's role in targeting specific PKA substrates, several lines of evidence indicate that gravin participates in

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PKA-dependent signaling events at or near the membrane. Tao et al. (2003) showed that gravin-PKA interaction facilitates PKA phosphorylation of both β2-adrenergic receptor (β2AR) and gravin itself. Gravin and β2AR phosphorylation enhances their physical interaction with one another, which regulates receptor resensitization following isoproterenol-induced desensitization (Tao et al., 2003). A study by Havekes et al. (2012) also showed reduced β2AR phosphorylation in gravin knockdown mice and implicated a role for gravin in G protein switching (Havekes et al., 2012), which is another consequence of β2AR phosphorylation by PKA (Daaka, Luttrell, & Lefkowitz, 1997). Gravin may also target the phosphorylation of PDE4D, a PKA-enhanced phosphodiesterase that binds to gravin (Raymond, Wilson, Carter, & Maurice, 2007; Willoughby et al., 2006b). The finding that AKAR3 constructs targeted to the membrane show increased phosphorylation in the presence of gravin expands the possible range of PKA substrates targeted by gravin in this compartment. It would be interesting to explore the impact of gravin expression on other known PKA targets at or near the plasma membrane such as ion channels (Brown & Yule, 2010; Dai, Hall, & Hell, 2009; Swope, Moss, Raymond, & Huganir, 1999) and cytoskeletal regulators (Howe, Baldor, & Hogan, 2005; Y. Wang et al., 2006).

While the current study demonstrates that gravin potentiates PKA activity at the plasma membrane, our results also suggest that this potentiation is present within both raft and non-raft membrane microdomains. Evidence for this was seen in gravin's ability to direct the phosphorylation of two membrane-associated AKAR3 constructs: AKAR3-CAAX and Lck-AKAR3. AKAR3-CAAX contains a C-terminal prenylation domain that has been shown to localize preferentially at non-raft microdomains, whereas Lck-

AKAR3 contains a myristoylated and palmitoylated N-terminal domain has been shown to target ordered lipid domains known as membrane rafts (Agarwal et al., 2014; Allen & Zhang, 2006; Melkonian et al., 1999). While plasma membrane microdomains represent highly dynamic compartments, some evidence suggests that protein-protein interactions within these compartments can be exclusive (Depry et al., 2011; Gao et al., 2011; Zacharias et al., 2002). How then could gravin facilitate PKA dependent phosphorylation activity in different membrane microdomains? One possibility could be via dissociation and diffusion of C subunits from R subunit dimers as described by the classical model of PKA activation. However, serious challenges to this dissociation model have been outlined in recent years. First, cAMP binds to PKA with low diffusion constant on the order of nanomolars, so it is not likely to be readily turned over in cells (Poppe et al., 2008). In addition, Smith et al. (2013) show that AKAP18-PKA complexes – similar to gravin-PKA in that both involve RIIα – retain the catalytic subunit (PKAc) even after isoproterenol stimulation and subsequent elevation of PKA activity. In fact, Smith et al. (2013) further showed that a flexible linker domain on RIIα works to constrain PKAc to substrates within a ~16 nanometer radius (F. D. Smith et al., 2013). In addition to these important studies, the current study also challenges the PKAc dissociation model, evident in the finding that gravin was able to reduce cytosolic PKA activity through interaction with PKA and with the plasma membrane. This finding would be difficult to explain were PKAc to dissociate into the cytosol following forskolin treatment (an example of subcellular PKAc dissociation is seen in Martin, Deerinck, Ellisman, Taylor, & Tsien, 2007). Therefore, the extent of gravin-directed PKA activity at the membrane may more likely due to the localization of gravin within both raft and non-raft microdomains. To be

sure, the localization of gravin within these microdomains is currently not well understood; however its N-myristoylation motif suggests a preferred localization within membrane rafts. This is in part supported by a study by Choi et al (2008), which showed that plasma membrane cholesterol extraction in SNU-449 cells caused gravin redistribution away from the cell periphery to intracellular vesicles (M. C. Choi, Lee, Kim, Lee, et al., 2008). In a more recent study, Su et al (2013) showed that exogenous rodent gravin, also called SSeCKS, was found in caveolin-rich membrane fractions corresponding to membrane rafts. However, Su et al. also showed moderate gravin labeling in "lighter" membrane fractions which supports the notion that gravin may localized within both membrane microdomains, at least in exogenous expression models (Su et al., 2013). The current study also uses exogenous gravin expression, so it is unclear whether levels of endogenous gravin would result in a similar localization within both raft and non-raft membrane microdomains, or facilitate the phosphorylation of both membrane-targeted AKAR3 constructs as observed in the current study. Nonetheless, if the breadth of PKA activity across membrane microdomains is proportional to the level of gravin expression in cells, this may serve as an important regulatory mechanism for PKA phosphorylation at the plasma membrane. This would be a fascinating topic of further research, especially given that gravin expression is highly regulated in cells under various physiological conditions such as cancer (reviewed in Gelman, 2012), inflammation (Kitamura et al., 2002; Sun, Cheng, Liu, Xiao, et al., 2007; Q. H. You et al., 2010), tissue injury/stress (Chen et al., 2008; Dolinay et al., 2006; Higgins et al., 2003), and hypoxia (Wasenius et al., 2003; Weissmuller et al., 2014).

The finding that gravin localization at the cell periphery is impaired by the mutation of a consensus binding motif for Ca^{2+}/calmodulin (CaM) within the CB4 domain suggests a novel role for CaM interaction with gravin. A previous study by Tao et al. (2006) suggests that CaM plays a role in relocalization of gravin to the cytosol upon calcium elevation by binding to the membrane-associated polybasic domains, PB1-3 (Tao et al., 2006). More recently, we demonstrated that a gravin mutant missing PB1-3 still underwent calcium-dependent redistribution identical to that of full-length gravin, which suggests that the role of CaM in mediating gravin redistribution is more complex than previously thought. In fact, deletion of gravin's CB4 domain – a fourth putative CaM binding domain located downstream of gravin's polybasic domains – caused a significant reduction in the basal localization of gravin the cell periphery even in the absence of calcium elevation (Schott & Grove, 2013). Since CaM has been linked to gravin redistribution, we proposed in the previous study that deletion of the CB4 domain might mimick a CaM-gravin interaction and prevent gravin from associating with the plasma membrane. However, as the current study indicates, a gravin construct with mutations to the CaM-binding amino acid sequence within the CB4 domain caused a similar reduction in membrane localization as CB4 deletion, suggesting that CaM-CB4 interaction promotes gravin localization at the plasma membrane under basal conditions. Thus, while CaM binding to gravin may regulate the dissociation of gravin from plasma membrane, possibly via an interaction with polybasic domains 1-3, CaM binding may also regulate the association of gravin at the membrane through binding to CB4. At the moment, it is not clear how CaM binding to the CB4 domain might support membrane association. One possibility is that the CB4 domain may support the myristoylation site in binding to the

membrane through an as yet unknown mechanism. Previous work by us shows that the myristoylation site is sufficient to retain normal membrane localization in the absence of a region containing the polybasic domains (X. Yan et al., 2009), but it is not sufficient for normal gravin-membrane localization in the absence of a region containing both the polybasic domains and CB4 (Schott & Grove, 2013). Perhaps in the absence of CaM-CB4 binding, the conformation of gravin's tertiary structure renders the myristoylation site and PB1-3 inaccessible to membrane phospholipids. It is also interesting that the CB4 domain (a.a. 669–693) lies directly upstream of three PKA-phosphorylation sites (ser696-698) which have been shown by Tao et al. (2003) to enhance gravin's association with β2AR. It is possible that CaM binding to CB4 may influence either the phosphorylation/dephosphorylation of serines 696-698 or the interaction of these sites with other proteins. Future studies could investigate the importance of gravin's CB4 domain in serine 696-698 phosphorylation and in β2AR interaction to gain clues into the role of this domain in membrane localization.

Demonstration that thapsigargin pre-treatment abolished gravin's ability to potentiate PKA activity at the plasma membrane indicates a role for gravin in regulating cross-talk between calcium- and cAMP-dependent signaling pathways. Several lines of evidence clearly show that although gravin is distributed at the plasma membrane under basal conditions, gravin redistributes away from the membrane in response to PKC activation (Lin et al., 1996a; Piontek & Brandt, 2003) and/or cytosolic Ca^{2+} elevation (Tao et al., 2006), both of which are also shown to cause the redistribution of PKA away from the cell periphery (Schott & Grove, 2013; X. Yan et al., 2009). Despite the understanding that gravin redistribution alters PKA localization, the impact of gravin

redistribution on subcellular PKA activity has not been demonstrated until now. In the current study, pre-treatment with thapsigargin caused the redistribution of gravin and prevented gravin-mediated potentiation of PKA activity at the plasma membrane. Importantly, thapsigargin pre-treatment had no significant impact on membrane PKA activity in the absence of gravin, demonstrating that the impact of thapsigargin on membrane PKA activity was gravin-dependent and likely mediated by gravin redistribution. The notion that calcium elevation impacts PKA activity through changes in gravin distribution represents a novel mode of calcium/cAMP crosstalk. It is well documented that crosstalk between these two second messenger pathways has broad physiological relevance, and several pathways have been described. Calcium-dependent signaling is known to regulate cAMP production through various adenylyl cyclase isoforms, but calcium can also regulate cAMP degradation by stimulating type 1 phosphodiesterase (reviewed in Halls & Cooper, 2011). The current study describes a mechanism of Ca^{2+}/cAMP crosstalk downstream of cAMP production and degradation that operates through the spatial regulation of PKA by gravin. This lays groundwork for future studies to investigate the impact of gravin-mediated calcium/PKA crosstalk *in vivo*. For example, based on the current study we predict that calcium impacts not only β2AR phosphorylation, but also plasma membrane PKA activity operating downstream of receptor activation. This may be important particularly in hippocampal neurons where gravin was shown to phosphorylate β2AR and regulate learning and memory (Havekes et al., 2012). Gravin is also shown to regulate cellular migration, a phenomenon which relies heavily on the compartmentalization of PKA at the leading edge, and also on calcium/PKA crosstalk (Howe, 2011). Since our knowledge of gravin-dependent PKA

phosphorylation targets remains somewhat limited, it is hard to predict the precise impact of gravin in the context of calcium signaling. Proteomic knowledge of PKA substrate dynamics in the presence and absence of gravin may be critical in future studies.

Conclusions

We report here that gravin-V5/His expression impacted PKA activity at the plasma membrane and in the cytosol. This effect was seen in response to forskolin treatment, which showed higher levels of PKA activity at the plasma membrane and lower levels of PKA activity in the cytosol when gravin was expressed. Experiments using mutant gravin constructs revealed that this impact on plasma membrane and cytosolic PKA activity was dependent on gravin's interaction with PKA and localization at the cell periphery. Pretreatment with thapsigargin triggered the redistribution of gravin away from the cell periphery and inhibited gravin-V5/His from increasing forskolin-stimulated PKA activity at the plasma membrane. Our data shows that gravin expression may have profound effects on subcellular PKA activity and supports the hypothesis that gravin facilitates crosstalk between Ca^{2+} and cAMP-dependent signaling pathways.

Acknowledgements
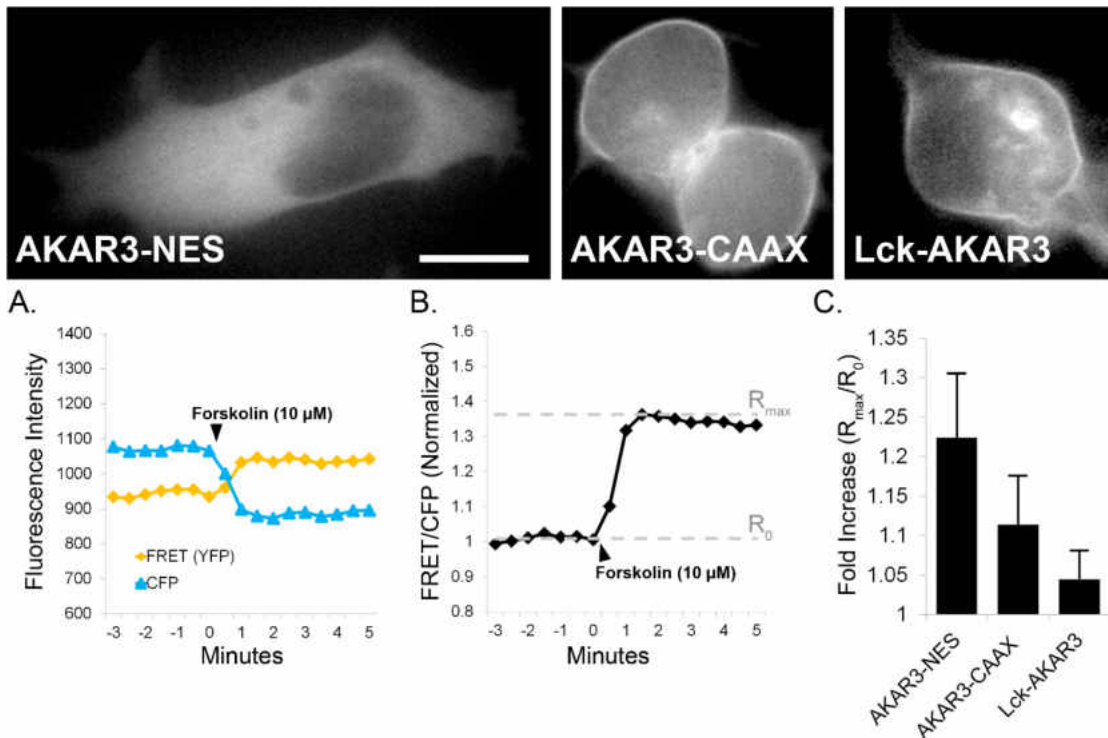
Figure IV-1. Quantification of PKA dynamics with AKAR3. Fluorescent images show
the distribution of three AKAR3 constructs that contain a targeting
sequence for the cytosol (NES), non-raft plasma membrane (CAAX), or
membrane rafts (Lck). Forskolin caused a drop in CFP fluorescence and
an increase in YFP fluorescence as imaged through CFP and FRET
channels, respectively. FRET intensity was corrected for CFP crossover
(44%) at each time point. The normalized ratio of FRET/CFP intensity
was then plotted over time, and the fold increase was calculated by
dividing the maximal ratio value after treatment (Rmax) by the average
ratio value prior to treatment (R0). Graph C represents the mean fold
increase in each of the AKAR3 constructs after 10 μM forskolin treatment.
Scale bar = 10 μm; cells were incubated in 10 μM IBMX for 30 minutes
prior to forskoliln treatment.
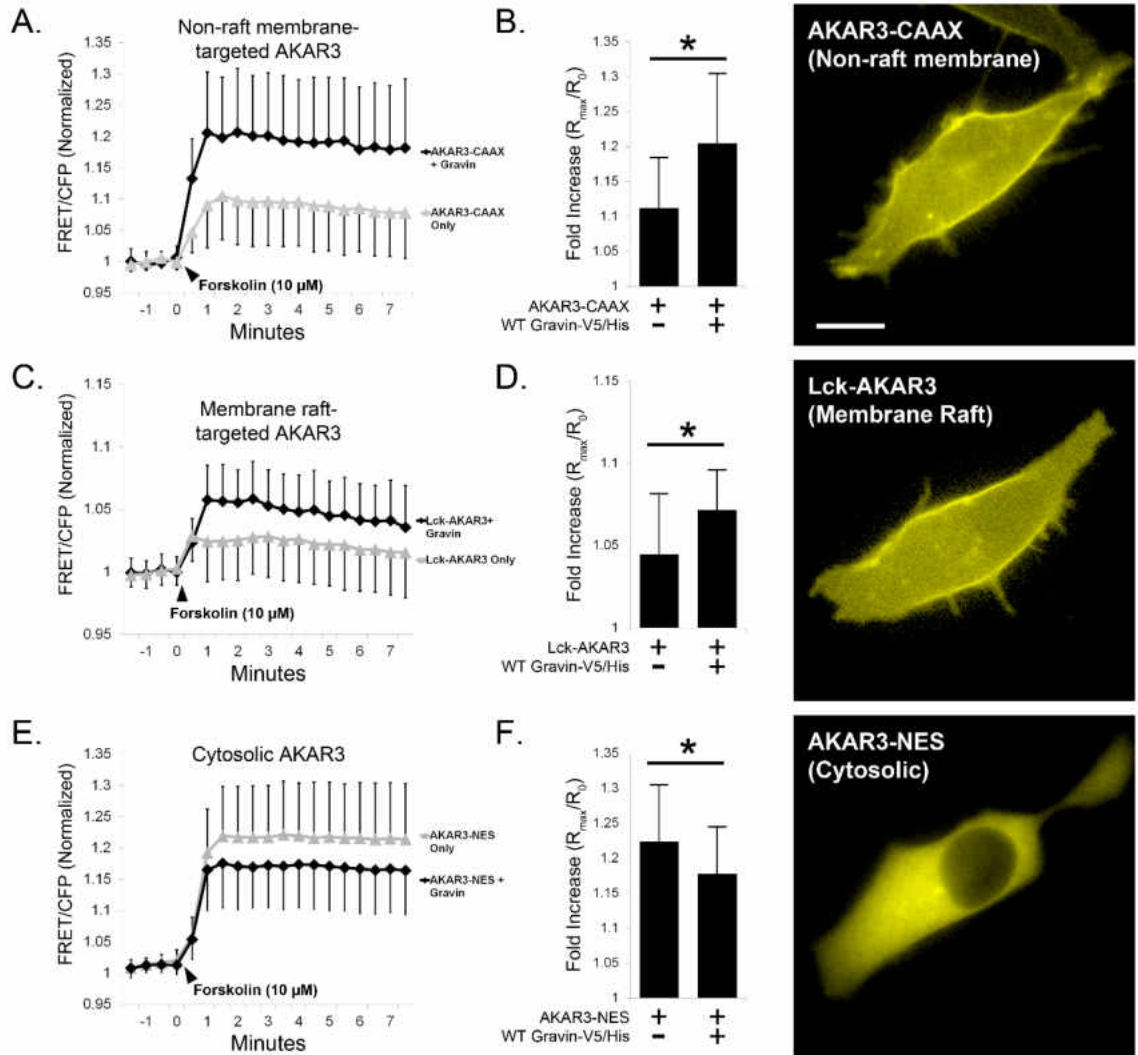
Figure IV-2. Gravin-V5/His expression alters subcellular PKA activity. AN3CA cells were co-transfected with gravin-V5/His and with AKAR3 constructs containing targeting sequences for non-raft membrane (CAAX), membrane raft (Lck), or cytosolic (NES) localization. Representative fluorescent images show the distribution the AKAR3 constructs. Expression of gravin increased forskolin-mediated PKA activity in both non-raft (A,B), and raft (C,D) membrane compartments. Conversely, gravin reduced PKA activity in the cytosol (E,F). Mann-Whitney Rank Sum tests revealed significant differences between the mean fold increases of each treatment. Asterisks denote $p < 0.05$. Scale bar = 10 μm ; cells were incubated in 10 μM IBMX for 30 minutes prior to forskolin treatment.

A. PKA localization at the membrane

WT Gravin-V5/His	+	−	−
(ΔPKA) Gravin-V5/His	−	+	−

B. Gravin expression in PKA RII-ECFP positive cells

WT Gravin-V5/His	+	−	−
(ΔPKA) Gravin-V5/His	−	+	−
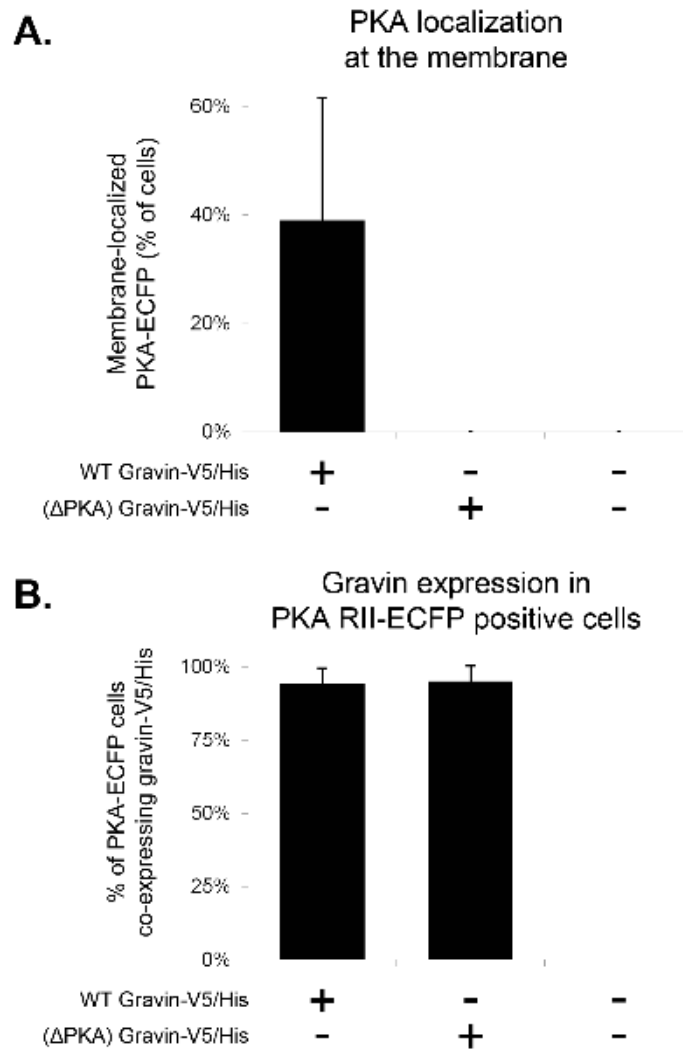
Figure IV-3. PKA RII-ECFP was localized to the cell periphery in AN3CA cells only when full-length WT gravin was present. Graph A shows that 39% of cells expressing PKA RII-ECFP showed ECFP localization at the cell periphery when co-transfected with WT gravin-V5/His. No cells showed PKA RII-ECFP localization at the cell periphery when co-transfected with (ΔPKA) gravin-V5/His, or when expressed in cells containing no gravin. Graph B shows the % co-transfection of PKA RII-ECFP positive cells with and without gravin-V5/His constructs. PKA-RII-ECFP positive cells showed a high rate of co-transfection with gravin-V5/His constructs as indicated by immunofluorescence labeling with a gravin antibody (94% with WT gravin-V5/His; 95% with (ΔPKA) gravin-V5/His), but cells transfected with PKA RII-ECFP only showed no immunofluorescence labeling the gravin antibody.
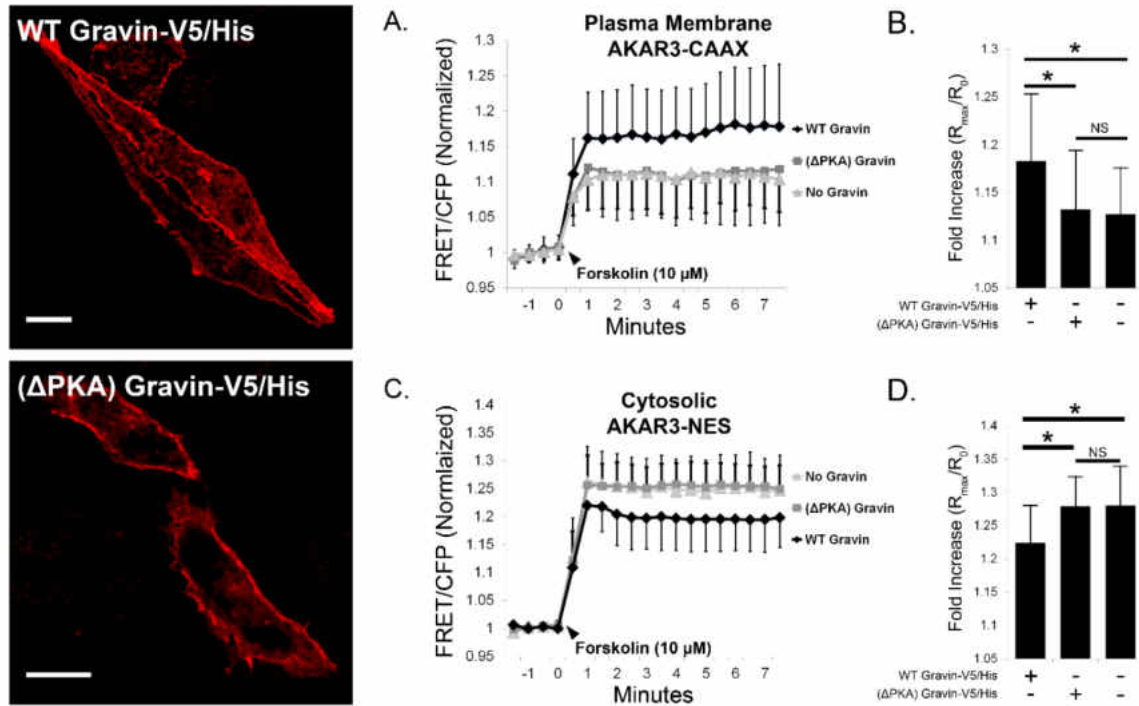
Figure IV-4.   Forskolin-stimulated PKA activity at the plasma membrane (AKAR3-CAAX) and cytosol (AKAR3-NES) in AN3CA cells co-expressing AKAR3 and either WT or (ΔPKA) gravin-V5/His. Graphs A,B show that while gravin expression caused an increase in forskolin-stimulated PKA activity at the plasma membrane, this increase was not observed in cells expressing ΔPKA gravin. Graphs C,D show that the gravin-mediated suppression of cytosolic PKA activity was not observed in cells expressing ΔPKA gravin. One-way ANOVA with Holm-Sidak post hoc tests revealed significant differences between the mean fold increase as indicated by asterisks. Representative confocal micrographs show the distribution of transfected gravin-V5/His constructs in fixed cells immunolabeled with a gravin antibody. Scale bar = 10 μm. Cells were incubated in 10 μM IBMX for 30 minutes prior to forskolin treatment.
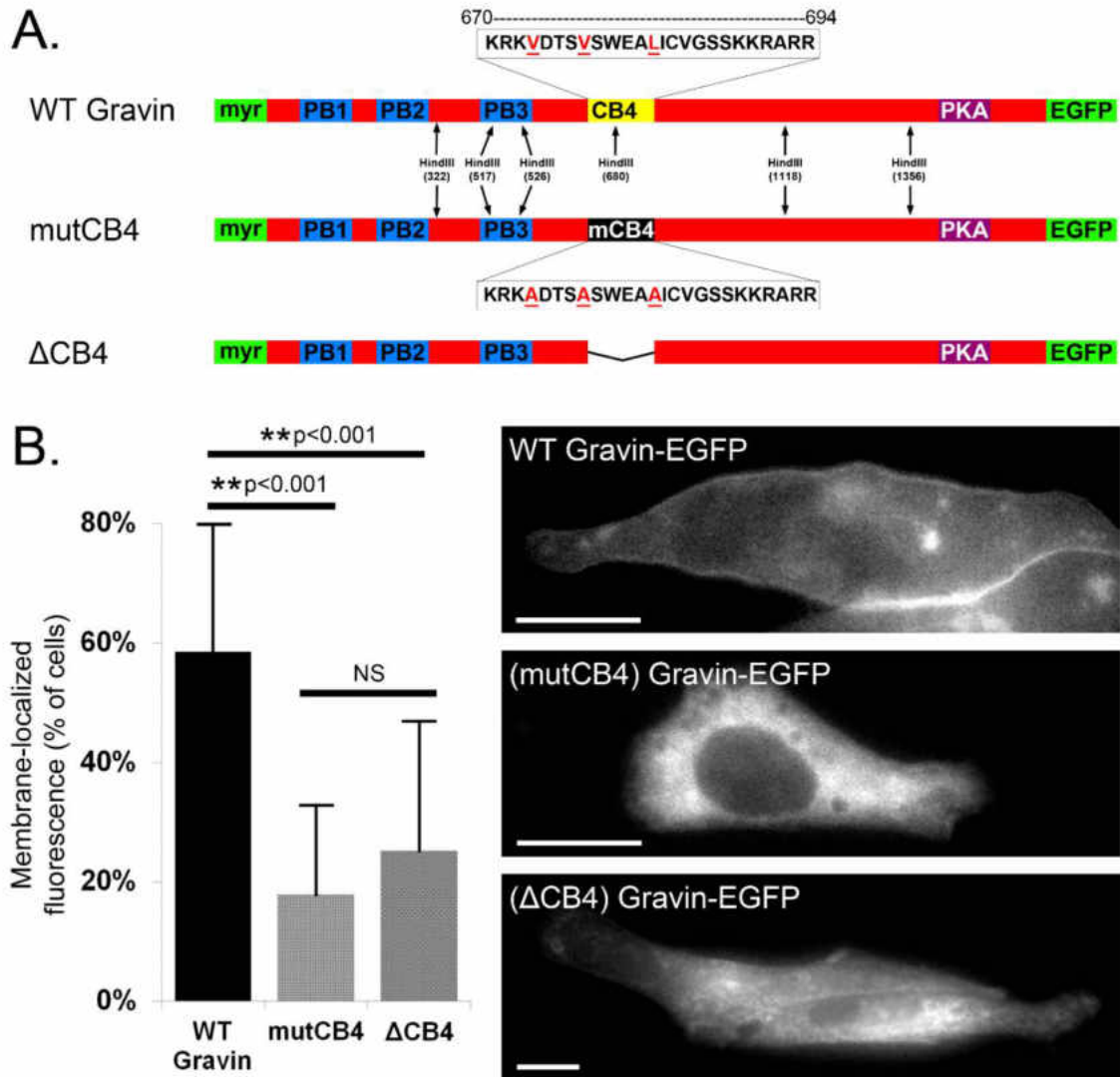
Figure IV-5.   Role of CB4 domain in subcellular gravin localization. Part A illustrates the sequence and location of gravin's CB4 domain downstream of the membrane targeting regions (myr, PB1-3). Amino acids shown in red comprise a calmodulin-binding consensus sequence and were substituted for alanine. Mutagenesis was confirmed by HindIII restriction digest (not shown). In graph B, either mutation or deletion of the CB4 domain caused a significant reduction in membrane localization compared to WT gravin. (ANOVA with Holm–Sidak post-hoc test; asterisks denote significant differences; NS, not significant). Representative fluorescent images show the localization of these constructs in AN3CA cells. Scale bar = 20 μm.
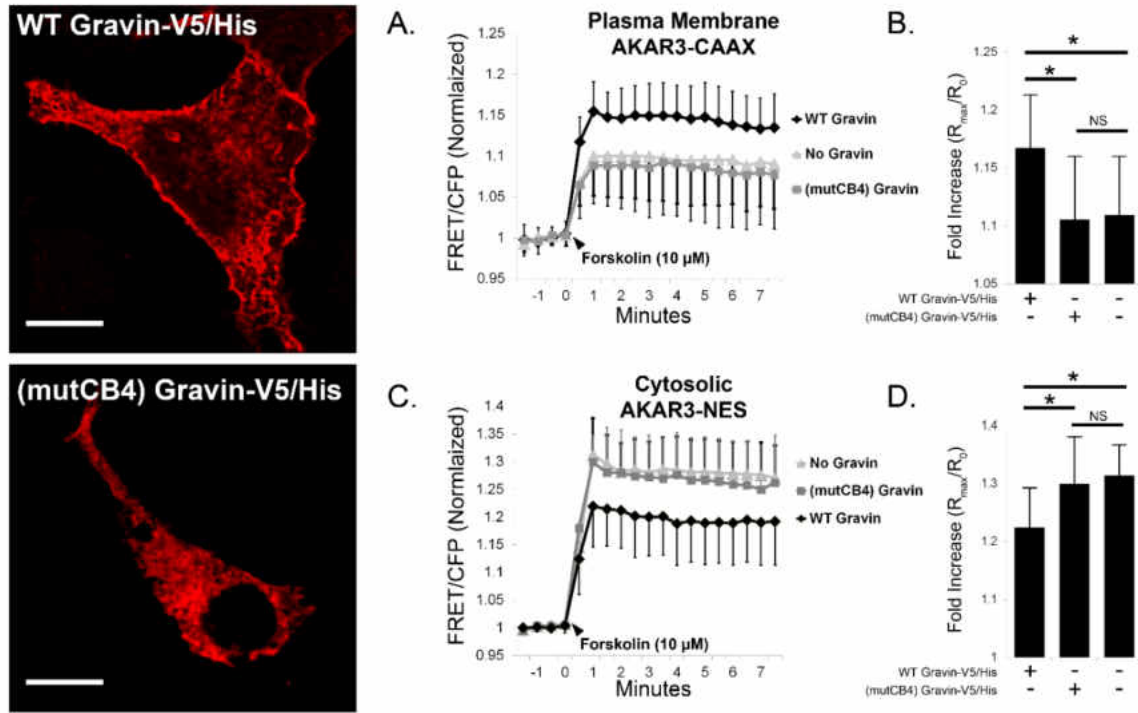
Figure IV-6.   Forskolin-stimulated PKA activity at the plasma membrane (AKAR3-CAAX) and cytosol (AKAR3-NES) in AN3CA cells co-expressing AKAR3 and either WT or (mutCB4) gravin. Graphs A,B show that while gravin expression caused an increase in forskolin-stimulated PKA activity at the plasma membrane, this increase was not observed in cells expressing mutCB4 gravin. Graphs C,D show that the gravin-mediated suppression of cytosolic PKA activity was not observed in cells expressing mutCB4 gravin. One-way ANOVA with Holm-Sidak post hoc tests revealed significant differences between the mean fold increase as indicated by asterisks. Representative confocal micrographs show the distribution of transfected gravin-V5/His constructs in fixed cells immunolabeled with a gravin antibody. Scale bar = 10 μm. Cells were incubated in 10 μM IBMX for 30 minutes prior to forskolin treatment.
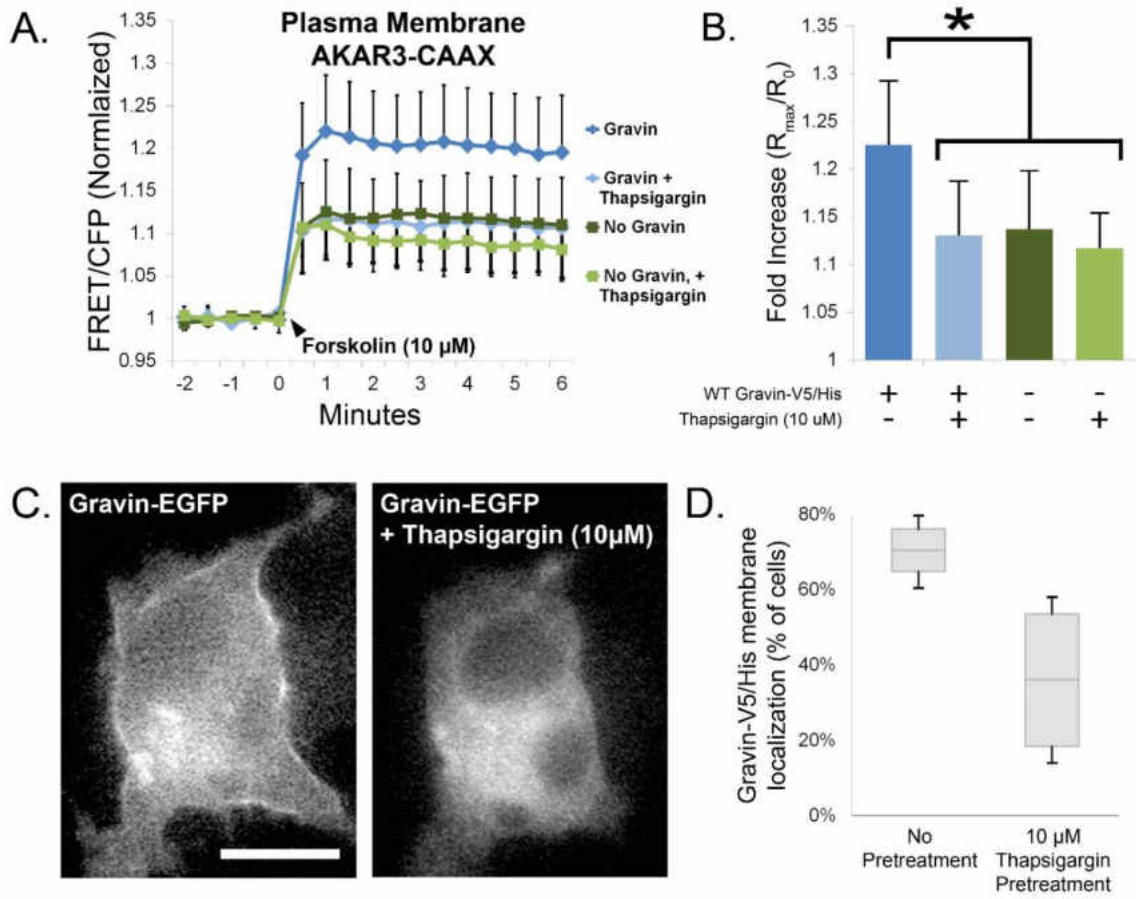
Figure IV-7.   Thapsigargin triggers gravin redistribution and abolishes gravin-mediated elevation in plasma membrane PKA activity. Graphs A,B show that gravin expression caused an increase in forskolin-stimulated PKA activity at the membrane, but this increase was not observed in cells expressing gravin but pretreated with the calcium-elevating agent thapsigargin (10 µm) for 30 min. Thapsigargin pretreatment had no effect on forskolin-stimulated PKA activity in the absence of gravin. One-way ANOVA on ranks with Kruskal-Wallis post hoc showed significant differences between treatments as indicated by asterisks. In part C, representative fluorescent images illustrate the redistribution of gravin-EGFP away from the plasma membrane by thapsigargin. Gravin-V5/His redistribution was confirmed after FRET experiments by quantifying the percentage of cells showing gravin localization at the membrane, shown in box plot represented in graph D. Scale bar = 10µm.

CHAPTER V

DISCUSSION

The goal of the current study was to investigate the hypothesis that gravin mediates crosstalk between calcium and cAMP dependent signaling pathways by translocating from the plasma membrane to the cytoplasm in response to intracellular calcium elevation. This study demonstrated that gravin undergoes redistribution in response to treatment with the calcium-elevating agents ionomycin, thapsigargin, and ATP. These calcium elevating agents triggered not only the redistribution of gravin but also the loss of compartmentalized PKA at the cell cortex. Calcium-mediated gravin redistribution did not require the calmodulin-binding polybasic domains (PB1-3), but a fourth calmodulin binding domain (CB4) was shown to be important for membrane localization under basal conditions and suggests that calmodulin plays a complex role in regulating gravin-membrane interaction. Gravin-membrane interaction was shown to be important for PKA localization and for compartmentalized PKA activity, as seen in studies using the PKA FRET biosensor AKAR3. These studies determined that gravin expression potentiates plasma membrane PKA activity but suppresses cytosolic PKA activity. Gravin's impact on membrane and cytosolic PKA activity were lost in cells expressing gravin constructs that either lacked the PKA binding domain or were inhibited in binding to the membrane. In addition, thapsigargin pretreatment, which caused gravin redistribution, prevented gravin-mediated potentiation of PKA activity at the plasma membrane. By demonstrating the impact of calcium-mediated gravin redistribution on

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PKA localization and subcellular activity, these studies support the hypothesis that gravin mediates crosstalk between calcium and cAMP dependent signaling pathways.

Gravin-mediated crosstalk between calcium and cAMP-dependent signaling outlined in the current study represents a novel mode of crosstalk between these two signaling pathways. The influence of calcium on cAMP-dependent signaling is well understood to occur in the regulation of cAMP levels themselves (Reviewed by Halls & Cooper, 2011). Production of cAMP begins with G-protein coupled receptors (GPCRs) that associate with trimeric G-proteins containing the stimulatory α-subunit, α_s. Upon GPCR activation, Gα_s dissociates from $\beta\gamma$ subunits and activates adenylyl cyclase (AC) enzymes, which then convert ATP to cAMP. Adenylyl cyclase activity (and therefore cAMP production) can be influenced by calcium-dependent signaling mediators which target certain isoforms in either a stimulatory or inhibitory manner. For example, Ca^{2+}/calmodulin (CaM) stimulates three of the ten known adenylyl cyclase isoforms, AC1, AC3, and AC9, whereas AC5 and AC6 are inhibited by CaM. Other adenylyl cyclase isoforms are further regulated by calcium-dependent phosphorylation by CaM-dependent kinases (CaMK) and dephosphorylation by CaM-dependent phosphatases (CaN). For example, AC1 and AC3 are inhibited by CaMK phosphorylation, and AC9 is inhibited by CaN dephosphorylation. In addition to calcium's impact on cAMP production, however, calcium-dependent signaling mediators also regulate cAMP degradation. For example, CaM is known to stimulate phosphodiesterase type 1, an enzyme that hydrolyzes cAMP back to AMP. It is clear from the current literature that calcium plays a complicated role in regulating intracellular cAMP levels, and the current study adds to this by showing that calcium exerts addition control over cAMP-dependent

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signaling downstream of cAMP production/degradation by regulating the interaction of

PKA with its targets (Fig. V-1). We report that this mechanism occurs through the

calcium-mediated redistribution of gravin. In our studies, gravin targeted PKA to the cell

periphery and potentiated forskolin-stimulated PKA activity at the plasma membrane. In

response to calcium elevation, gravin redistribution caused the relocalization of PKA

away from the cell periphery and also inhibited the potentiation of plasma membrane

PKA activity by gravin.
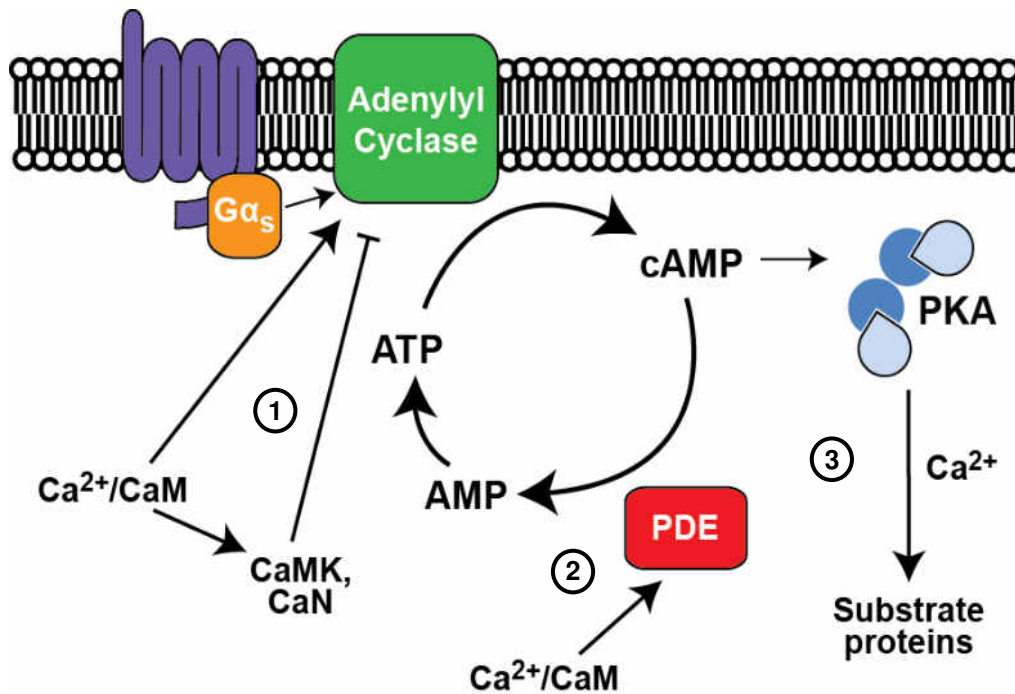
Figure V-1. Ca^{2+} crosstalk with cAMP signaling pathways occurs by (1)
positive/negative regulation of adenylyl cyclases which produce cAMP,
(2) regulation of phosphodiesterases that hydrolyze cAMP, and (3)
regulation of cAMP-dependent protein kinase (PKA) interaction with
substrate proteins, as evidenced by the current study.

Role of Calmodulin in Gravin-Mediated Ca^{2+}/cAMP Crosstalk

Future studies will be required to fully understand the mechanism behind calcium

-mediated gravin redistribution, but our results and others suggest a role for calmodulin in

this event. Tao et al. (2006) first asserted that CaM causes gravin redistribution by binding to the membrane associating polybasic domains (PB1-3) and thereby reversing their electrostatic interaction with the plasma membrane. However, a later study from our laboratory showed that a mutant gravin construct missing PB1-3 was still localized at the plasma membrane identical to full-length gravin due to the N-myristoylation site (X. Yan et al., 2009). This raised the interesting possibility that this mutant construct missing PB1-3 would not interact with CaM and would therefore remain at the plasma membrane upon calcium elevation. In the current study, we discovered that this was not the case. Even in the absence of PB1-3, gravin localized to the plasma membrane and translocated to the cytosol upon calcium elevation at an identical rate to that of full-length gravin, suggesting that the polybasic domains are in fact not necessary for calcium-mediated gravin redistribution. This result also supports another interesting finding by Yan et at. (2009) which showed that this same ΔPB1-3 construct also underwent PKC-mediated redistribution, despite a previous assertion that PKC phosphorylation of the polybasic domains mediates gravin redistribution. In search for an explanation for these results, we came to find that the ΔPB1-3 gravin mutant construct does retain a fourth putative calmodulin binding domain downstream of PB1-3. This region, which we have termed CB4, was first identified by Tao et al. (2006) and was reported to have no affinity for phospholipid vesicles. We therefore reasoned that the ΔPB1-3 grain mutant underwent calcium-mediated redistribution because of CaM interaction with the downstream CB4 domain. To test this assertion, we generated a gravin mutant missing PB1-3 and CB4, but surprisingly this mutant did not localize to the plasma membrane under basal conditions in a manner that was sufficient to measure it's redistribution upon calcium elevation. In

fact, both deletion of CB4 and mutation of the putative calmodulin binding sequence within the CB4 domain caused a dramatic reduction in gravin localization at the cell periphery. This finding suggests that while calmodulin interaction with PB1-3 could promote the dissociation of these domains from the plasma membrane, calmodulin interaction with CB4 promotes the association of gravin with the plasma membrane. This may provide insight not only into the membrane localization of gravin, but also into calcium mediated gravin redistribution.

It is possible that the function of gravin's CB4 domain is to support the membrane binding activity of the myristoylation site. Evidence for this comes from the finding that ΔPB1-3 gravin mutant is localized at the membrane, but the ΔPB1-3,CB4 gravin mutant is not, even though both of these mutant constructs contain the N-terminal myristoylation sequence. To put it another way, the myristoylation site is sufficient for membrane localization, but only when the CB4 domain is present. Although myristoyl lipid anchors are involved in the localization of proteins to plasma membranes, they are reported to have relatively weak binding energy by themselves and are thus supported either by additional lipid anchors like palmityol groups or by interactions with other membrane-bound or transmembrane proteins (Resh, 1999). Gravin does not contain any palmitoylation sequences, but the latter notion is quite possible since the CB4 domain (a.a. 669-684) falls within the β2AR binding region (a.a. 652-938) described by Tao et al. (2003). Based on this, it's possible that the CB4 domain itself and/or CaM binding to CB4 could play a role in facilitating gravin's interaction with β2AR, thereby supporting gravin's localization at the cell periphery. This is an exciting prospect that could be tested in several ways. The first would be to determine whether the interaction between full-

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length gravin and β2AR receptor is different than that of (mutCB4) gravin or (ΔCB4) gravin. Next, it should be confirmed whether calmodulin binds to CB4 and if this is important in gravin's interaction with β2AR. It would also be useful to explore gravin localization and interaction with β2AR receptor in living cells under calmodulin knockdown and overexpression conditions, or with pharmacological inhibitors of calmodulin such as Ophobolin-A, which prevents calmodulin from interacting with other proteins by covalently bonding to calmodulin's hydrophobic pocket. Gravin localization could be also assessed under β2AR receptor knockdown and overexpression conditions. Finally, it would be interesting to generate a peptide corresponding to the CB4 domain to serve as a competitive inhibitor for calmodulin interaction with this region. One could assess the impact of this peptide on (A) gravin localization at the cell periphery, and (B) gravin's interaction with β2AR. I predict that a CB4 peptide would competitively interfere with calmodulin binding to gravin and thus inhibit gravin's localization at the plasma membrane and possibly gravin's interaction with β2AR. This inhibitor peptide may even serve as a means for therapeutic intervention in regulating calcium and cAMP crosstalk in cells expressing gravin, which could be explored in vascular endothelial disorders and/or hippocampal learning and memory.

Ca^{2+}/cAMP Crosstalk in Vascular Function.

In the context of vascular endothelial cell function, unpublished work from our lab shows that gravin promotes cellular migration in a scratch wound healing model. In addition to this, gravin is concentrated at the leading edge of microvascular endothelial cell lamellipodia along with RhoA and F-actin. In light of the current study, gravin-mediated Ca^{2+}/cAMP crosstalk may be a promising avenue for research in the context of

endothelial cell migration. A review written by Alan Howe (2011) discusses the critical importance of calcium and PKA crosstalk in migrating cells, particularly with regard to localized calcium transients in at the leading edge (Howe, 2011). These transients are in part mediated by stretch-activated calcium channels (SACC) which become active in response to mechanosensation, causing transient extracellular calcium influx. TRPM7, for example, is known to produce calcium transients known as "flickers" in leading edge lamellipodia that are important for cellular migration. TRPC1 is another leading edge SACC that regulates cellular chemotaxis through lamellipodial calcium gradients. Finally, TRPV4 mediated calcium signaling is linked to cytoskeletal remodeling. The possible link between these SACC-mediated pathways and gravin in the context of endothelial cell migration are completely unknown but would make a fascinating subject for further research. It is possible that gravin's localization at the leading edge is mechanosensitive in response to SACC-mediated calcium entry. I predict that SACC channel activation would cause the transient redistribution of gravin away from the point of calcium influx, and this would additionally impact the interaction of PKA with downstream targets. Some of these targets may be cytoskeletal effector proteins such as RhoA, which is distributed at the leading edge with gravin in microvascular endothelial cells. Also, it is possible that a calmodulin-interfering peptide, such as the CB4 domain, could be generated to regulate gravin localization at the cell periphery and perhaps be used as a therapeutic agent to regulate endothelial cell migration. Given the clear link between gravin and endothelial barrier function and angiogenesis, this might create additional avenues for treatment of diseases such as stroke or tumor growth.

Another component of the vasculature that readily expresses gravin is smooth muscle cells, which utilize calcium signaling to regulate cytoskeletal organization and contractility. Bruce et al. (2003) reports an interesting smooth muscle phenomenon in which contractility and relaxation are both regulated by calcium but depend entirely on the shape of the calcium signal. For example, calcium sparks – localized calcium transients – mediate smooth muscle cell contractility while more global calcium signals mediate relaxation (Bruce, Straub, & Yule, 2003). While smooth muscle cells readily express gravin, the impact of gravin on smooth muscle function is poorly understood. It would be interesting to observe whether calcium sparks vs. whole cell calcium gradients impact gravin distribution, PKA distribution, and PKA activity/phosphorylation of downstream substrates.

Gravin is also present in cardiomyocytes of the heart, and knockdown of gravin in mice is shown to enhance cardiac contractility (Guillory et al., 2013). Given the central importance of calcium signaling in cardiac myocytes, it is possible that gravin mediates its effect on cardiac contractility in part through Ca^{2+}/cAMP crosstalk. Future experiments could be done to determine the difference in phosphorylation states of PKA substrates within wild-type versus gravin knockdown hearts, and assess how calcium signaling in cardiac myocytes may regulate this phosphorylation in a gravin-dependent manner. In addition, calcium signaling in cardiac myocytes may regulate β2AR receptor sensitivity by affecting its association with gravin.

<center>Role of Gravin in GPCR Crosstalk</center>

The current study suggests that gravin intersects with GPCR signaling in a number of ways. This line of investigation also represents a promising next step for

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further research. This study showed that receptor-mediated signaling linked to $[Ca^{2+}]_i$ elevation and PKC activation caused gravin and PKA redistribution. In addition, gravin redistribution was shown to regulate not only PKA localization, but gravin-mediated potentiation of PKA activity the cell periphery. Given these findings, it is reasonable to hypothesize that (1) gravin potentiates Gs-coupled GPCR signaling at the plasma membrane, (2) Gq-coupled GPCRs impact cAMP-dependent signaling through gravin redistribution, and therefore (3) gravin mediates crosstalk between Gq-coupled and Gs-coupled GPCRs.

Evidence that gravin may potentiate GPCR-stimulated PKA activity at the plasma membrane comes from the demonstration that gravin directs PKA localization at the cell periphery through its PKA-binding domain (also shown by X. Yan et al., 2009) and that gravin potentiates plasma membrane PKA activity upon treatment with forskolin, an agent which raises cAMP levels by stimulating adenylyl cyclase. GPCRs coupled to Gs are known to stimulate adenylyl cyclase and raise cAMP levels, suggesting that these receptors may also be subject to gravin-mediated potentiation of PKA activity at the plasma membrane. An obvious context for the investigation of this hypothesis would be within β2AR receptor signaling, since gravin is known to physically interact with this Gs-coupled receptor (G. F. Fan et al., 2001; Shih et al., 1999b). This, however, brings up an interesting question: Does gravin impact PKA activity in only certain Gs-coupled GPCR pathways or does it regulate a wide array of Gs-coupled GPCR pathways? This question could be investigated by treating cells with different Gs-coupled GPCR agonists in cells expressing AKAR3 biosensors in the presence and absence of gravin. This approach could also be investigated in the context of a physiological output, such as endothelial

barrier function. A recent study by Weissmuller et al. (2014) showed that gravin supports endothelial barrier formation in response to treatment with PKA-stimulating GPCR agonists albuterol (β-adrenergic agonist) and NECA (adenosine receptor agonist). Perhaps gravin accomplishes this by potentiating PKA activity at the plasma membrane in response to these agonists.

In addition to gravin's potential impact on Gs-coupled GPCR systems, it is also likely that Gq/11-coupled GPCRs may cause gravin redistribution, thereby altering PKA localization and compartmentalized PKA activity. Evidence for this comes from the findings that ATP caused gravin and PKA redistribution in HEC1A cells (Fig. III-8), and that gravin redistribution required both calcium elevation and PKC activity (Fig. III-7). Since ATP-mediated gravin redistribution was unaffected in the absence of extracellular calcium, it is likely that this change occurred through P2Y receptors, many of which are coupled to Gq/11. Given that Gq/11-coupled GPCRs are known to cause both PKC activation and $[Ca^{2+}]_i$ elevation from intracellular stores through phospholipase C activity, it is possible that other Gq/11-coupled GPCRs also cause gravin redistribution and alter PKA localization. To test this, one could treat cells expressing gravin-EGFP with a variety of agonists that target Gq/11-coupled GPCRs and measure the impact on gravin distribution. These experiments could also be applied to AKAR3-expressing cells in the presence and absence of gravin to measure the impact of GPCR-mediated gravin redistribution on forskolin-stimulated PKA activity at the plasma membrane (see Fig. IV-5). Going back to the example of endothelial barrier function, it's interesting to note that some of the most potent agonists for endothelial permeability – thrombin, bradykinin, and histamine – target Gq/11-coupled GPCRs (Mehta & Malik, 2006). Given gravin's role in

supporting endothelial barrier integrity (Weissmuller et al., 2014), perhaps these agonists stimulate endothelial permeability in part by redirecting PKA away from the cell periphery through gravin redistribution.

Gravin's putative interplay with these various GPCR pathways raises the interesting possibility that gravin may be involved in a crosstalk mechanism between Gq/11-coupled and Gs-coupled GPCR pathways. One very interesting line of investigation might be found within GPCR families that couple to both G protein pathways through various receptor classes, as is the case with purinergic P2Y receptors and with adrenergic receptors. In these examples, both of the mentioned GPCR pathways can be activated by a common agonist. ATP activates P2Y receptors, most of which are coupled to Gq/11 (Erb et al., 2006). However ATP can also stimulate Gs-coupled pathways through $P2Y_{11}$ (Qi, Kennedy, Harden, & Nicholas, 2001) or adenosine receptor activation if extracellular ATP becomes metabolized to adenosine through surface-expressed ectonucleotidases (Narravula, Lennon, Mueller, & Colgan, 2000). In the case of adrenergic receptors, epinephrine/norepinephrine activates both Gq/11-coupled α1 adrenergic and Gs-coupled β-adrenergic receptors. Within both adrenergic and purinergic systems, one could test how Gs-coupled GPCR signaling is impacted by the presence of Gq/11-coupled GPCRs, and whether gravin affects this interplay. I predict that Gq/11 activation would trigger gravin/PKA redistribution and thereby impact Gs-mediated subcellular PKA activity. This could very likely be the case in hippocampal neurons, which express both gravin and multiple adrenergic receptor subtypes and have already been linked in functions related to learning and memory (Havekes et al., 2012). Gravin may be involved in regulating postsynaptic inputs through an interplay between dendritic

GPCRs, or be more broadly involved in dendritic Ca^{2+} and PKA signaling as is the case

with AKAP79 (Dell'Acqua et al., 2006; Fuller, Fu, Scheuer, & Catterall, 2014; Gomez,

Alam, Smith, Horne, & Dell'Acqua, 2002; Murphy et al., 2014; Oliveria, Dell'Acqua, &

Sather, 2007; Oliveria, Dittmer, Youn, Dell'Acqua, & Sather, 2012). To test this idea, one

could begin with a thorough characterization of gravin expression in the hippocampus to

identify both the types of hippocampal neurons that express gravin and also the

subcellular localization of gravin within these neurons. Gravin redistribution could also

be assessed following stimulation of Gq/11-coupled GPCRs, such as α1-adrenergic

receptors. If α1ARs trigger gravin redistribution through Ca^{2+} and/or PKC mediated

pathways, it would be interesting to determine how this could impact β2AR signaling and

the interaction of β2AR with gravin. Knockdown or pharmacological inhibition of α1AR

and β2AR could also be performed to determine how these receptors might affect one

another upon norepinephrine stimulation in the presence of gravin.

<div align="center">Conclusions</div>

In conclusion, the current study demonstrates that gravin mediates crosstalk

between calcium-dependent and cAMP-dependent signaling pathways. Under basal

conditions, gravin localizes PKA to the cell periphery which results in the potentiation of

plasma membrane PKA activity and the suppression of cytosolic PKA activity.

Intracellular calcium elevation causes gravin redistribution to the cytosol, which triggers

both the loss of PKA compartmentalization and loss of PKA potentiation at the plasma

membrane. Calcium therefore exerts control over cAMP-dependent signaling pathways

by altering the subcellular compartmentalization of PKA through gravin redistribution.

Although the physiological impact of this crosstalk mechanism is currently unclear,

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future research could lead to translational findings toward the treatment of disease. First, a more detailed understanding of the mechanism behind gravin redistribution might lead to pharmacological tools to regulate gravin distribution *in vivo* without the manipulation of specific signaling networks. Second, many facets of vascular function could be pursued in understanding gravin-mediated Ca^{2+}/cAMP crosstalk including endothelial cell migration, smooth muscle function, and cardiac contractility. Finally, knowledge of gravin's precise interplay between different GPCR-based signaling networks could be usefully applied to other health and disease models, such as endothelial barrier function and hippocampal learning and memory. The current study gives strong justification for the exploration of gravin function within these contexts and paves the way for novel therapeutic strategies and a more robust understanding of the complexity of cellular signal transduction.

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ABBREVIATIONS

α1AR	α1-adrenergic receptor
AKAP	A-Kinase Anchoring Protein
AKAR3	A-Kinase Activity Reporter 3
ATP	adenosine triphosphate
β2AR	β2-adrenergic receptor
BAPTA-AM	1,2-Bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetrakis(acetoxymethyl ester)
BIM	bisindolylmaleimide
$[Ca^{2+}]_i$	intracellular calcium concentration
CAAX	C-terminal prenylated domain
CaM	calcium-calmodulin
cAMP	cyclic adenosine monophosphate
CB4	calmodulin binding domain 4
ECFP	enhanced cyan fluorescent protein
EGFP	enhanced green fluorescent protein
EYFP	enhanced yellow fluorescent protein
FRET	Förster's Resonance Energy Transfer
Fsk	Forskolin
GPCR	G protein coupled receptor

IBMX	3-isobutyl-1-methylxanthine
IM	ionomycin
InsP3	inositol triphosphate
kD	kilodaltons
Lck	N-terminal myristoylated and palmityolated domain
myr	myristoylation site
NES	nuclear export signal
PB1-3	polybasic domains 1 through 3
PDE4	phosphodiesterase type 4
PKA	protein kinase A
PKAc	catalytic subunit of PKA holoenzyme
PKC	protein kinase C
PLC	phospholipase C
RII	regulatory subunit of type II PKA
SERCA	sarco/endoplasmic reticulum calcium ATPase
SES	standard extracellular solution
SOCE	store-operated calcium entry
SSeCKS	Src-Suppressed C-Kinase Substrate
Tg	thapsigargin
WT	wild-type/full-length