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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 300 kD 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 s 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.

1. 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 [1]. 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 [2], 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 [3]). Numerous reports demonstrate that the loss of PKA compartmentalization significantly disrupts PKA signaling and leads to many physiological dysfunctions, for example, in memory [4], immune response [5], and cytoskeletal dynamics [6].

The AKAP12 gene encodes a 300 kD AKAP known as gravin in humans [7,8] or SSeCKS in rodents [9], that binds the RII subunit of PKA and is expressed in a variety of cell types and tissues [10]. Three

Abbreviations: AKAP, A-Kinase Anchoring Protein; 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; CaM, calcium–calmodulin; CB4, calmodulin binding domain 4; ECFP, enhanced cyan fluorescent protein; EGFP, enhanced green fluorescent protein; EYFP, enhanced yellow fluorescent protein; GPCR, G protein coupled receptor; IM, ionomycin; InsP3, inositol triphosphate; PB1–3, polybasic domains 1 through 3; PDE4, phosphodiesterase type 4; PKA, protein kinase A; 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.

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isoforms of gravin have been described (gravin-α, -β, -γ) which differ in subcellular localization [11]. 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 [12,13]. Like many AKAPs, gravin is a multivalent scaffold and interacts not only with PKA, but also with PKC [8,14,15], phosphodiesterase 4D [16], Ca^{2+}/calmodulin [12], and the β2-adrenergic receptor [17–20]. 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 [21–24]. Along similar lines, Choi et al. [25] 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 [26]. 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 [17–20]. Willoughby et al. [16] 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 to occur in response to PKC activation by phorbol ester treatment [15,27]. 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 [13]. 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) [12]. 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 [13]. 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 $[Ca^{2+}]_i$ 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 demonstrated 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 demonstrated 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.

2. Material and methods

2.1. Cell culture and transfection

AN3 CA cells and HEC-1-A cells (Manassas, VA, ATCC numbers: HTB-111, HTB-112 respectively) were cultured 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 100 μg/ml streptomycin. Growth medium was replaced three times each week, and cells were split 1:25 upon reaching confluence.

For experiments involving transfected expression vectors, cells were transfected 2–3 days after placing 2×10^4 cells/cm^2 onto 25 mm glass coverslips in an 8-well plate. Cells were incubated in a transfection solution containing 3 μl/ml GeneJammer (Agilent) and 1 μg/ml plasmid DNA for approx. 12–24 h prior to microscopy.

2.2. Construction of gravin–EGFP expression vectors

Gravin–EGFP, (ΔPB1–3) gravin–EGFP, and PKA RII ECFP constructs were initially constructed as described in previous work [13]. The current study used four additional gravin constructs which were generated as follows. A gravin–EYFP construct lacking nucleotides encoding the PKA RII binding domain (Δ4621–4662) termed "(ΔPKA) gravin–EYFP" was generated using a Phusion® site-directed mutagenesis kit (New England Biolabs, product F-541; forward primer 5′ACAGCCGTTGACCAGTT TGTACGTACAGAA; reverse primer 5′TTCCAAAATCCCATTTTCAGGCT CTAAATC). A gravin–EGFP vector lacking nucleotides encoding the three polybasic domains and the downstream putative calmodulin binding domain (Δ125–2086) 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′CCAAAGC[CCGCGG]TGGATACCTCAGTATCT; 5′GGTGCGTC AAAGTCGGCTACGGGGGTGC, mutagenic nucleotides underlined, constructed SacII sequence in brackets). A gravin–EGFP vector lacking nucleotides encoding 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′GCAAGGAGAAGT[CCGCGG] CTGATGAGGAA; 5′GGTGCGTCAAAGTCGGCTACGGGGGTGC, mutagenic nucleotides underlined, constructed SacII sequence in brackets). Finally, a gravin–EGFP construct lacking nucleotides encoding 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).

2.3. Western blotting

To obtain protein samples for Western blotting, transfected AN3 CA cells in T-25 flasks 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