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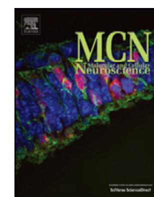

Regulated endosomal trafficking of Diacylglycerol lipase alpha (DAGLα) generates distinct cellular pools; implications for endocannabinoid signaling

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ABSTRACT

Diacylglycerol lipase alpha (DAGLα) generates the endocannabinoid (eCB) 2-arachidonylglycerol (2-AG) that regulates the proliferation and differentiation of neural stem cells and serves as a retrograde signaling lipid at synapses. Nothing is known about the dynamics of DAGLα expression in cells and this is important as it will govern where 2-AG can be made and released. We have developed a new construct to label DAGLα at the surface of live cells and follow its trafficking. In hippocampal neurons a cell surface pool of DAGLα co-localizes with Homer, a postsynaptic density marker. This surface pool of DAGLα is dynamic, undergoing endocytosis and recycling back to the postsynaptic membrane. A similar cycling is seen in COS-7 cells with the internalized DAGLα initially transported to EEA1 and Rab5-positive early endosomes via a clathrin-independent pathway before being transported back to the cell surface. The internalized DAGLα is present on reticular structures that co-localize with microtubules. Importantly, DAGLα cycling is a regulated process as inhibiting PKC results in a significant reduction in endocytosis. This is the first description of DAGLα cycling between the cell surface and an intracellular endosomal compartment in a manner that can regulate the level of the enzyme at the cell surface.

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1. Introduction

The diacylglycerol lipases (DAGLα & DAGLβ) are the enzymes that synthesize 2-arachidonylglycerol (2-AG), an endogenous ligand for the cannabinoid receptors types 1 and 2 (CB1 and CB2). DAGL-dependent endocannabinoid (eCB) signaling regulates synapse formation during development by modulating axonal growth and guidance and, in the postnatal brain, regulates neural stem cell proliferation and neuroblast migration (Oudin et al., 2011a; Oudin et al., 2011b; Zhou et al., 2015). At developed synapses this pathway also regulates synaptic plasticity via retrograde signaling (Ohno-Shosaku and Kano, 2014). In the above context, DAGLα and the CB1 receptor co-localize in axonal growth cones in developing neurons but whereas the CB1 receptor remains expressed presynaptically at developed synapses, DAGLα becomes restricted to the postsynaptic dendritic spine (Bisogno et al., 2003; Uchigashima et al., 2007; Williams et al., 2003; Yoshida et al., 2006). This selective localization of DAGLα to dendritic spines in developed neurons highlights how the regulated compartmentalization of this enzyme can determine eCB function.

Knockout (KO) mice studies revealed that depolarization-induced suppression of inhibition (DSI) and depolarization-induced suppression of excitation (DSE) are lost in hippocampus, cerebellum and striatum in DAGLα −/− mice (Gao et al., 2010; Tanimura et al., 2010). The modulation of synaptic strength by 2-AG regulates many behaviors including appetite, pain and cognition (Alger and Kim, 2011) and DAGLα and CB1 receptor KO mice share the same disrupted phenotypes (Powell et al., 2015). The adverse psychiatric consequences of inhibiting CB1 function in humans (Moreira and Crippa, 2009) are also likely to be consequential to the disruption of 2-AG signaling at synapses as DAGLα KO mice have been used to demonstrate a causative link between synaptic eCB signaling and anxiety and depressive behaviors (Shonesy et al., 2014). Thus, it is important to understand how DAGLα is trafficked in the dendrites and the cell body of neurons and other cells.

A ~370 amino acid C-terminal tail differentiates DAGLα from DAGLβ (Reisenberg et al., 2012). Members of the Homer family of adaptor proteins that can anchor binding partners to the postsynaptic density (PSD) interact with a defined motif within the tail, and DAGLα can be co-immunoprecipitated with Homer 2 in Neuro-2a cells (Jung et al., 2007). DAGLα also co-immunoprecipitates with the calcium/calmodulin-dependent protein kinase II (CaMKII) via an interaction with the tail and this kinase is also enriched within the PSD (Shonesy et al., 2013). Thus a number of mechanisms are emerging that might to some extent govern the molecular interactions of DAGLα at the PSD,

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but these do not speak to the dynamics of DAGLα trafficking to and within dendritic spines.

The dendritic spine has long been recognized as a highly dynamic structure, with changes in both spine morphology and molecular composition of the postsynaptic membrane impacting on synaptic plasticity (Anggono and Huganir, 2012; Hoogenraad and Bradke, 2009). The location of DAGLα within the dendrites and cells will determine not only where 2-AG can be made, but might also govern how 2-AG is released. With this in mind we embarked on a study to determine to what extent DAGLα exists in a dynamic as opposed to a static pool at the cell surface in dendrites and in cells. Our results have revealed for the first time that DAGLα can cycle within endosomes between an intracellular pool and a cell surface pool in both neuronal dendrites and COS-7 cells, the latter in a PKC-dependent manner. This newly discovered dynamic trafficking of DAGLα points to new sites of 2-AG synthesis and provides for a mechanism to regulate surface levels of the enzyme. Both are likely to impact on eCB signaling.

2. Results

2.1. Design and verification of the epitope-tagged DAGLα constructs

The DAGLα protein consists of a short intracellular N-terminal (22 amino acids) and four transmembrane domains (green), followed by a catalytic domain (pink) and a relatively long (~370 amino acids) tail at the C-terminus (shown schematically in Fig. 1A). The extracellular loops are poorly conserved and have no obvious binding function (Reisenberg et al., 2012). We therefore designed a construct expressing human DAGLα with a HA tag inserted in the first extracellular loop, retaining an intracellular V5 tag located on the C-terminal of a previously described DAGLα construct (Bisogno et al., 2003). The correct insertion of the HA sequence was confirmed by sequencing and Western blot; furthermore, immunostaining analysis of a number of cell types showed the construct to be expressed in a manner that was not obviously different from constructs lacking the extracellular tag. The specificity of staining the V5 tag was proved by expressing the construct in COS-7 cells; here V5 antibody staining was not detected in untransfected cells and could only be detected in cells that express the transgene once they had been permeabilised (data not shown). The HA-DAGLα construct was then transfected into cultured hippocampal neurons and the expression detected on the cell surface of live cells with an *anti*-HA antibody two days after transfection. The distribution of cell surface DAGLα pool was similar to the total DAGLα distribution as revealed by the V5 tag labeling, exhibiting a clear punctate pattern in the dendrites (Fig. 1B, C), with extensive overlap confirmed by colocalization analysis (Fig. 1D). However, importantly, some V5-positive puncta were not labeled with HA, suggesting that DAGLα may be present in intracellular compartments (arrowheads in Fig. 1C).

To reveal the synaptic localization of surface DAGLα, we triple labeled the transfected neurons with the above tags and an antibody to Homer 1, a canonical PSD marker (Shiraishi-Yamaguchi and Furuichi, 2007). There was extensive co-localization between DAGLα on the dendritic surface and Homer (Fig. 1B, C, D). These results show that the surface pool of DAGLα in the dendrites of hippocampal neurons is located either at or in near vicinity of the PSD, mirroring the presynaptic distribution of the surface CB1 (Dudok et al., 2015).

2.2. DAGLα is constitutively internalized and recycled in hippocampal neurons

We next tested the fate of the cell surface pool of DAGLα. To this end, hippocampal neurons transfected with HA-DAGLα were live-labeled for HA and allowed to internalize the label for 20 min. The cultures were then fixed and stained for the antibody/DAGLα complex at the cell surface and then permeabilized to stain for any internalized antibody/DAGLα complex (see Materials and methods for details). As before, two pools of DAGLα were detected, a surface pool and an internalized pool in the cell body as well as in the neuronal dendrites (arrowheads in Fig. 2A).

In order to follow the fate of the internalized DAGLα, a recycling assay was carried out (see Materials and methods for details). Briefly, cells were allowed to internalize the label as above and then subjected to an “acid wash” to remove surface-bound antibody whilst leaving the internalized antibody-receptor complexes intact (Fig. 2B). If the internalized antibody/DAGLα complex is retained within the cell, the antibody will not reappear on the cell surface. In contrast, the reappearance of the label at the cell surface would indicate recycling back to the cell surface. After 30 min, the HA label was again readily detected at the cell surface (Fig. 2C). Thus, DAGLα is constitutively internalized and recycled back to the cell surface in neurons.

2.3. DAGLα is constitutively internalized and recycled in COS-7 cells

For further study of DAGLα trafficking, we turned to COS-7 cells as a model system due to their ease of culturing and morphology favoring imaging studies. DAGLα on the surface of COS-7 cells was labeled in live cell cultures as before and returned to the incubator at 37 °C for various times to allow endocytosis. As in neurons, internalization of surface DAGLα resulted in its accumulation in intracellular punctate structures, indicating that DAGLα is constitutively internalized in the COS-7 cells (Fig. 3A). Internalization was evident after 20 min and increased over time at a steady rate (Fig. 3B).

The next step was to test if antibody/DAGLα complex can be recycled back to the cell surface. Cells expressing the DAGLα construct were live-labeled and then either washed and fixed directly or acid washed before fixation (Fig. 4A, B). The acid wash step again completely stripped the HA-antibody from the cell surface (compare Fig. 4A and B). As expected, internalized antibody/DAGLα complexes can readily be detected after incubating the cells at 37 °C for 30 min and acid wash to strip the surface bound antibodies provided the cells are permeabilized (Fig. 4C). The next step was to test if antibody/DAGLα complex can be recycled back to the cell surface. Cells that had been antibody fed and incubated for 30 min at 37 °C were acid washed and returned to the incubator for another 30 min. At this time point antibody/DAGLα complexes were again readily detected at the cell surface (Fig. 4D). Therefore, as with neuronal dendrites, DAGLα can be internalized and recycled back to the cell surface in COS-7 cells.

2.4. Internalized DAGLα co-localizes with microtubules but not F-actin

Based on cell staining it is apparent that the cell surface pool of DAGLα, as well as the intracellular pool, can be seen in highly organized domains. Depending on the plane of focus the staining is either highly punctuate, and/or tubular suggesting localization within a reticular structure (e.g. see Fig. 3A). Based on this observation, we looked for co-localization of DAGLα with cytoskeleton markers (phalloidin for F-actin, and antibodies against β-tubulin for microtubules). Confocal images revealed extensive co-localization between DAGLα and β-tubulin close to the plasma membrane (Fig. 5A), however, co-localization with F-actin was very limited and/or less obvious (Fig. 5B). Analysis of the images confirmed that the correlation of the intensities (shown as Pearson's R value) of DAGLα and β-Tubulin was significantly higher than the value from DAGLα and F-actin (Fig. 5C).

2.5. Internalized DAGLα was transported to EEA1 and Rab5-positive early endosomes via clathrin-independent endocytic pathway

To investigate the intracellular itinerary of the internalized DAGLα, we first sought to identify the endocytic pathway involved in its internalization. Clathrin-mediated endocytosis (CME) represents the best characterized pathway for internalizing membrane glycoproteins, but

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