CHEMICAL REVIEWS

REVIEW

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Regulation and Functions of Diacylglycerol Kinases

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1. OVERVIEW OF DIACYLGLYCEROL KINASES (DGKS)

Diacylglycerol (DAG) is an important lipid that is both an intermediate in lipid biosynthetic pathways and can act as a signaling lipid. The majority of signaling DAG is generated by hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP_2) by the enzyme phospholipase C (PLC), but DAG can also be generated when phosphatidic acid phosphatases remove the phosphate headgroup from phosphatidic acid (PA) (Figure 1). Signaling DAG serves to activate or recruit to membranes a number of proteins, including protein kinase C (PKC) isoforms, Ras-guanyl-nucleotide-releasing proteins (RasGRP), and chimaerins, which are Rac GTPase activating proteins (GAPs). Like DAG, PA is an intermediate in lipid biosynthetic pathways and can also activate several signaling proteins. Because of their importance, it is crucial that the intracellular levels DAG and PA be tightly regulated, which is accomplished by the diacylglycerol kinases and phosphatidic acid phosphatases. As such, these enzymes have numerous important functional roles.

1.1. Unique Aspects of DGKs from Multicellular Organisms

DGKs have been identified in most organisms that have been studied, and it appears that they have gained specialization in more complex species. For example, bacteria express only one DGK, which is an integral membrane protein capable of phosphorylating DAG and other lipids such as ceramide. This DGK does not appear to have structural elements that allow regulation of its activity, indicating that the limiting factor is access to its substrates. A recently identified eukaryotic enzyme that similarly phosphorylates several different lipid substrates, called multisubstrate lipid kinase (MuLK)[1] or acylglycerol kinase (AGK),[2] is not structurally similar to the DGKs and is not included in this review.

1.2. Mammalian Isoforms of DGK

Unlike bacteria and yeast, multicellular organisms express more than one, and often several, DGK isoforms that can be grouped by common structural elements into five subfamilies (Figure 2). The DGKs expressed in mammals are the best characterized, and 10 of them have been identified.[3−5] Like DGKs in other multicellular organisms, all of the mammalian DGKs have two common structural features: at least two cysteine-rich C1 domains and a catalytic domain. The C1 domains are homologous to the DAG-binding C1A and C1B motifs of PKCs,[6] but the C1 domain closest to the catalytic domain has an extended region of 15 amino acids not present in

Special Issue: 2011 Lipid Biochemistry, Metabolism, and Signaling

Received: December 1, 2010
Published: July 29, 2011

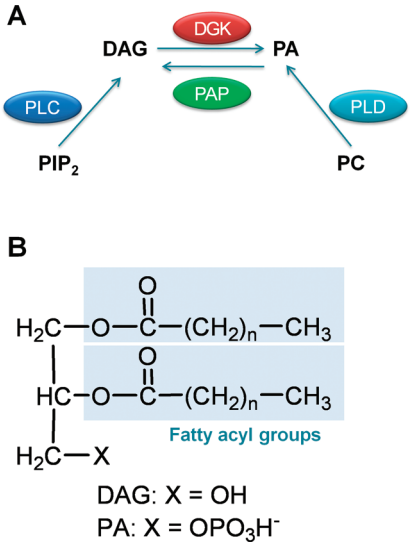

Figure 1. (A) Different enzymatic pathways can produce diacylglycerol (DAG) and phosphatidic acid (PA). PLC enzymes generate DAG that can be phosphorylated by DGKs to produce PA. In another pathway, phospholipase D (PLD) hydrolyzes phosphatidylcholine (PC) or phosphatidylethanolamine making PA that can be further hydrolyzed by PAPs to generate DAG. To date, there is little definitive evidence to suggest that the DGK and PAP reactions are coupled. Both DAG and PA can act as signaling lipids and are also intermediates in lipid biosynthetic pathways. (B) DAG and PA have the same general structure but contain different functional groups attached to the third (*sn*-3) carbon. Depending on their molecular structure, the fatty acyl groups confer different signaling properties to the DAG or PA.

C1 domains from other proteins or in the other C1 domains of DGKs. Mutations within this extended region significantly reduced kinase activity, indicating that this extension appears to contribute to DGK activity.[7] In theory, DGK C1 domains bind DAG, perhaps localizing them to where DAG accumulates. However, it is still controversial whether all DGK C1 domains can bind DAG or whether only some of them are capable of binding this lipid. Of several that were tested, only the C1 domains of DGKs β and γ could bind DAG analogues (phorbol esters), while the C1 domains of DGKs δ, η, and θ did not bind.[8−10] These results were in agreement with sequence alignments performed by Hurley and colleagues,[6] who predicted that only the C1 domains from DGKs β and γ could bind DAG while other DGK C1 domains were sufficiently different from those in PKCs that they might not bind DAG. Supporting the possibility that DGK C1 domains might serve alternative functions, the C1 domains of some DGKs, like those in other proteins, can act as protein−protein interaction sites. Indeed, the C1 domains of DGKζ associate with β-arrestins,[11] and they bind directly to Rac1.[12] It will be enlightening to test the phorbol ester binding capacity of all DGK C1 domains and to solve their crystal structures so that we can understand the differences between the C1 domains of DGKs and other proteins that contain them.

The catalytic domains in DGKs are composed of accessory and catalytic subunits. In most cases, these subunits are joined to create an uninterrupted catalytic domain. However, in the type II DGKs δ, η, and κ,[4,13,14] these domains are separated by a long peptide sequence that does not have any apparent functional motif. Each catalytic subunit has an ATP binding site where mutation of a glycine in this motif to an aspartate or alanine renders the DGK kinase dead.[15−17] Evidence suggests that some DGK catalytic domains may also require other motifs for maximal activity because catalytic domains from DGKs ε, ζ, and θ have very little DGK activity when expressed as isolated subunits (M.K.T. and R. M.E. unpublished observations and ref 7), although the isolated catalytic domain of DGKα retained about 1/3 the activity of a fully active DGKα N-terminal truncation mutant.[10] Thus, it appears that unlike bacterial DGK, several mammalian DGK catalytic domains require other motifs for maximal activity. It is possible that these other motifs somehow function in coordination with the catalytic domain.

In addition to the C1 and catalytic domains, DGKs contain other structural domains that form the basis of the five subtypes. In general, these other domains help regulate the level of kinase activity or the localization of the enzyme or both. For example, type I DGKs, α, β, and γ, have calcium-binding EF hand motifs that make these enzymes more active in the presence of calcium. Evidence from mutational studies indicates that when the EF hand motifs of DGKα bind calcium, a conformational change occurs that allows membrane association and activation of the enzyme.[18] Type II DGKs, δ, η, and κ, have pleckstrin homology (PH) domains near their amino termini. This domain in DGKδ has been shown to bind weakly and non-selectively to phosphatidylinositols,[19,20] but binding these lipids did not significantly affect its activity.[19] DGKs δ and η also have a sterile alpha motif (SAM) at their carboxy termini. A recent study shows that SAM domains of DGKδ bind zinc at multiple sites and might allow DGKδ to form oligomers.[21] A mutant of DGKδ containing a SAM domain refractory to zinc binding exhibits partially impaired localization to the cytoplasmic puncta and enhanced localization to the plasma membrane in response to TPA stimulation, thus suggesting that zinc may play an important role in the assembly and physiology of DGKδ.[21] There is also further evidence that SAM domain interactions sequester DGKδ away from membranes to limit its access to diacylglycerol.[22]

The only type III DGK, ε, does not have identifiable structural motifs outside of its C1 and catalytic domains. But despite its lack of identifiable regulatory domains, this is the only DGK that displays specificity toward acyl chains of DAG; it dramatically prefers DAGs with an arachidonoyl group at the *sn*-2 (middle) position of the glycerol backbone. Its preference for arachidonoyl-DAG suggests that DGKε may be a component of the biosynthetic pathway that accounts for the enrichment of PIP_2 with arachidonic acid.[23] This possibility is discussed in more detail below. Type IV DGKs, ζ and ι, have a motif enriched in lysines and arginines that acts as a nuclear localization signal and is a substrate for conventional PKCs. This motif is homologous to the phosphorylation site domain of the myristoylated alanine-rich C kinase substrate (MARCKS) protein, and phosphorylation of this domain limits nuclear localization of these DGKs. The ζ and ι DGK isoforms also have four ankyrin repeats and a PDZ binding motif at their carboxy termini that may be sites of protein−protein interactions. The only type V DGK, θ, is distinguished by three C1 domains, a PH domain, and a Ras-association domain within the PH domain. To date, no binding partners for the PH and Ras-association domains have been identified.

1.3. Alternative Splicing

Six of the ten mammalian DGK isoforms (DGKs β, γ, δ, ζ, η, and ι) have been demonstrated to undergo alternative splicing

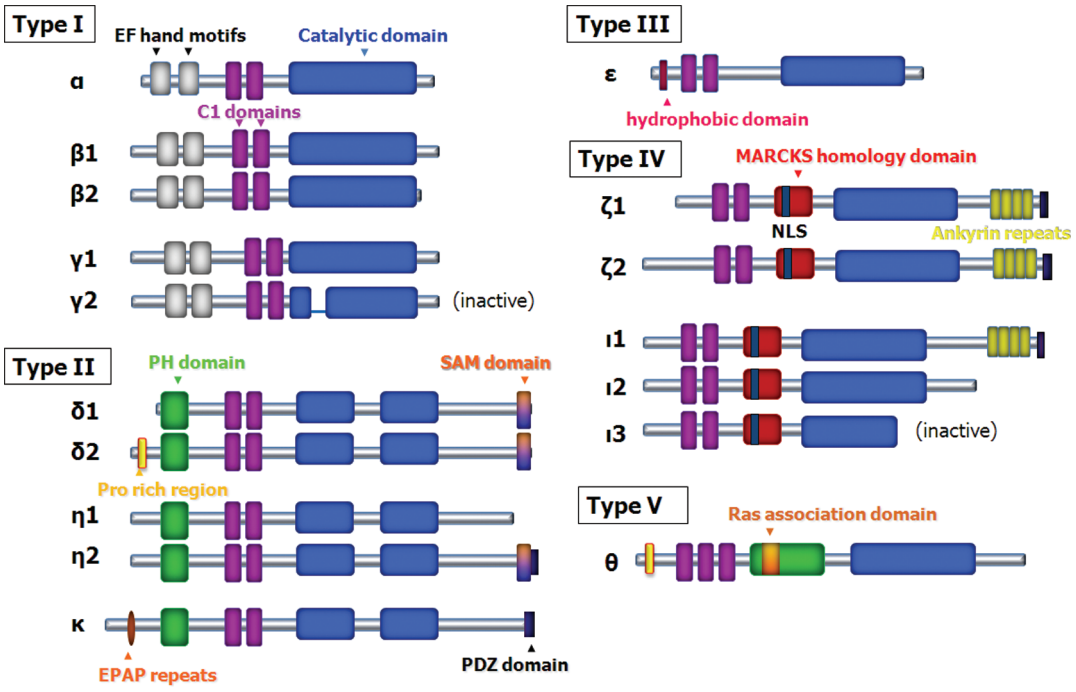

Figure 2. The mammalian DGK family. Based on structural motifs, the 10 mammalian DGKs are divided into five subtypes. Alternative splicing of some DGK isotypes generates even more structural diversity. Alternative splicing variants are designated by a number following the Greek letter. Many of the DGKs contain other unique structural domains that are not shown.

(Figure 2). In most cases, this splicing alters either the localization or the activity levels of the enzymes. Two of the three type I enzymes are alternatively spliced. DGKβ, for example, has two splice variants that differ at their carboxy -termini.[24] These two splice variants have similar activity, but the shorter variant of DGKβ does not translocate to the plasma membrane following growth signals or treatment with phorbol esters, while the longer variant does. The physiological significance of this alternative splicing is not clear. DGKγ is also alternatively spliced,[25] with the shorter splice variant missing a portion of its catalytic domain. As expected, this truncation renders the enzyme inactive. The mRNA encoding both DGKγ splice variants was detected in several tissues, while only the active, longer variant was detected in brain and retina. Again, the physiological significance of this splicing is not clear, but it suggests that there is a biological role for kinase inactive DGKγ.

Type II DGKs also undergo alternative splicing. DGKδ, for example, is variably spliced at its amino terminus.[26] DGKδ2, the longer variant, is widely expressed in both normal tissues and various cancer cell lines, while DGKδ1, the shorter variant, has a more limited spectrum of expression. The amino terminus of DGKδ2 is proline-rich, and this region appears to impede translocation of the enzyme to the plasma membrane upon exposure of cells to phorbol esters. In contrast, DGKδ1, which does not have the proline-rich amino terminus, rapidly relocates from the cytoplasm to the plasma membrane upon exposure of cells to phorbol esters. Phorbol esters also have opposing effects on the expression levels of endogenous DGKδ splice variants: they increase the levels of DGKδ2 and reduce the levels of DGKδ1. Activation of the epidermal growth factor receptor (EGFR) similarly induces expression of DGKδ2, indicating that this splice variant might be an important regulator of signals downstream of the EGFR. Another type II DGK, DGKη, is also alternatively spliced, which results in one variant (DGKη2) having a SAM domain while the other variant (DGKη1) lacks it.[27] DGKη1 is expressed in most tissues and cancer cell lines, while DGKη2 has a more limited expression pattern. In addition, DGKη1 was found to have higher specific activity compared with DGKη2, and domain swapping experiments suggested that the SAM domain might serve to negatively regulate DGKη2, possibly through an indirect effect of binding to another SAM domain.[27] This domain also appeared to promote prolonged retention of DGKη2 on endosomes following exposure of cells to osmotic or oxidative stress. Finally, the abundance of the DGKη splice variants was differentially regulated by glucocorticoids and phorbol esters: both agents augmented expression of DGKη1 mRNA and reduced the expression of DGKη2.[27]

Both type IV DGKs undergo alternative splicing. At its amino terminus, the gene encoding DGKζ is spliced to form a longer variant, DGKζ2, which is predominantly expressed in skeletal muscle.[28] The shorter variant, DGKζ1, is expressed in most tissues. Both splice variants have similar activity levels, but DGKζ2 appears to be predominantly localized in the nucleus while DGKζ1 is found both inside and outside of the nucleus. Their distinct localization patterns likely dictate their ability to regulate RasGRP1, which is discussed in more detail below. The other type IV DGK, DGKι, is differentially spliced to form three variants.[29] One variant (DGKι1) is the full length enzyme, another (DGKι2) lacks the ankyrin repeats, and the third variant (DGKι3) is truncated within the catalytic domain. DGKs ι2 and ι3 are predominantly associated with membrane compartments, while the ankyrin repeats retained in DGKι1 appear to help localize a portion of the enzyme in the cytoplasm. In addition to affecting localization, the splicing also alters the specific activity levels of the enzymes: DGKι1 has the highest activity levels, DGKι2 has about 50% of that activity, and DGKι3, as expected, is

dx.doi.org/10.1021/cr1004106 |*Chem. Rev.* 2011, 111, 6186–6208

inactive. Like splicing of other DGK isoforms, the biological function of alternative splicing of DGKι is not clear.

Given the extensive alternative splicing that is known to occur within mammalian DGK genes, it would not be surprising if other DGK genes not discussed above are also differentially spliced. Clearly, the diversity of the DGK family is enhanced by alternative splicing, but the functions of the differentially spliced variants needs to be studied in much more detail.

1.4. Organ Distribution

Most tissues express several different DGK isoforms, and even within the same cell type, more than one DGK isoform can exist. For example, we detected all known DGK isoforms in mouse brain extracts[30] and have found expression of at least six DGK isoforms in mouse embryo fibroblasts (M.K.T. unpublished observations). In general, when several DGKs are expressed in tissues or cells, they are from different subfamilies, suggesting that each subfamily carries out a distinct biological function. But because no one has assayed the relative expression levels of the DGK family in a systematic way, it is difficult to directly compare the expression levels of DGK isoforms in each tissue. However, one way to compare the levels of DGKs in tissues is to examine the frequency at which cDNA clones, called expressed sequence tags (ESTs), of each DGK are identified in cDNA libraries prepared from different tissues. This information is available at the National Center for Biotechnology Information (NCBI, www.ncbi.nlm.nih.gov), which collects EST profile data. It should be noted that these data only approximate the levels of DGK mRNA in a given tissue and are not meant to be definitive. Given that caveat, this database suggests that most tissues express at least one member of each DGK subfamily, with brain and hematopoeitic organs particularly enriched in DGKs. The EST data also suggests that DGKs α and ζ are the most commonly expressed isoforms, with both of them being expressed in almost every tissue examined. Conversely, DGKs β, κ, and ι are expressed at much lower levels and in fewer tissues compared with other DGK isoforms. DGKβ, for example, is expressed predominantly in nerves and brain, indicating an important role for this isoform in neural tissue. Because of the fairly ubiquitous expression patterns of most DGK isoforms, it is perhaps more interesting to consider outliers in this data set such as tissues that express a single DGK isoform or only a few DGKs. The limited DGK expression profile in these tissues probably suggests that the DGKs that are expressed have particularly important functions. For example, DGKε is the only DGK isoform that has been identified in adipose tissue, DGKγ is the only isoform isolated from pituitary tissue, only DGKs α and θ have been identified in bone marrow, and DGKs α, δ, and ζ are the most abundant isoforms in lymphocyte-rich tissues such as lymph nodes, spleen, and thymus. Alternatively, no DGK ESTs have been isolated from parathyroid tissue, suggesting that DGK activity might be dispensable in parathyroid glands. While identifying the tissue distribution of each DGK is enlightening, it is perhaps more informative to understand the specific function of each DGK in these tissues by studying their subcellular distribution, their modes of regulation, and their physiological roles in model systems. These issues are discussed in detail below.

1.5. Subcellular Distribution

DGKs have been identified in a number of cell compartments, including the nucleus (Figure 3). Their localization within the nucleus is not surprising because it has a phosphatidylinositol cycle that is regulated separately from plasma membrane

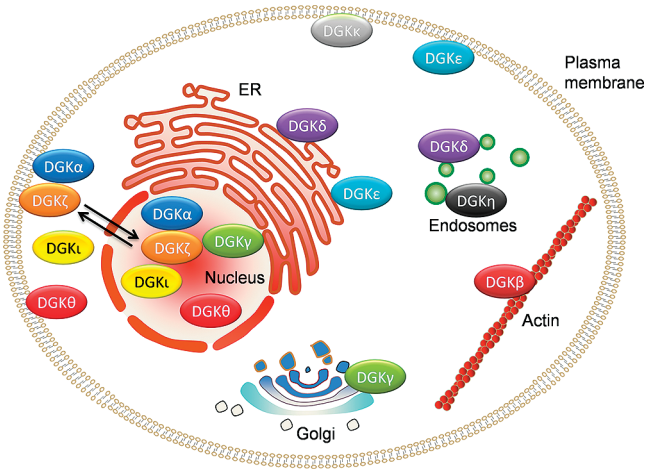

Figure 3. Cell distribution of mammalian DGKs.

phosphatidylinositol signaling.[31] Diverse extracellular stimuli such as growth factors can lead to generation of nuclear DAG in temporally distinct patterns,[32,33] and several groups have demonstrated that nuclear DAG fluctuates during the cell cycle.[31] Nuclear DAG appears to have important functional roles, so it is not surprising that several DGKs have been detected in the nucleus. DGKs α, ζ, and ι shuttle into and out of the nucleus,[17,34−36] while a significant fraction of DGKθ localizes there constitutively.[37] These nuclear DGKs appear to be confined to separate, distinct regions of the nucleus: DGKs θ, ζ and ι have been identified in discrete, unidentified regions within the body of the nucleus,[17,34,37,38] while DGKα appeared to predominantly localize around its periphery.[35] Their distinct intranuclear localization patterns might be expected because nuclear DAG signaling is likely compartmentalized as independently fluctuating pools that have distinct fatty acid compositions.[39] The specific nuclear roles of DGKs α and ζ are discussed in detail in section 3.1.

In addition to localizing within the nucleus, DGKs are also found throughout other parts of the cell (Figure 3). Most of them are at least partly localized at the plasma membrane either constitutively, in the case of DGKκ,[4] or following stimulation with specific agonists. For example, DGKα translocates to the plasma membrane following engagement of the T cell receptor,[40] DGKδ1 translocates there upon exposure to phorbol esters,[26] and DGKs ζ and θ are found at the plasma membrane following activation of some G protein-coupled receptors.[41,42] Presumably, their function at the plasma membrane is to attenuate DAG signaling initiated by specific receptors, several examples of which are discussed in section 1.6.

In addition to the plasma membrane, DGK activity has been detected in cell fractions containing cytoskeleton components along with other proteins involved in cytoskeleton dynamics.[43] Consistent with this, DGKθ was found to associate with RhoA,[44] DGKβ colocalized with actin filaments,[45] and DGKζ interacted with several proteins involved in actin dynamics.[3] In most cases, the physiological significance of their interactions with cytoskeleton components is not entirely clear, but there are data demonstrating that DGKs can modulate cytoskeleton remodeling. For example, DGK inhibitors augmented platelet secretion and aggregation,[46] and DGKζ is involved in actin dynamics,[47] which is discussed in more detail in section 3.2. DGKs have also been found to colocalize with organelles. DGKγ, for example,

dx.doi.org/10.1021/cr1004106 |Chem. Rev. 2011, 111, 6186–6208

colocalizes with the Golgi,[45] DGKs ε and δ appear to reside in the endoplasmic reticulum,[45,16] and DGKs δ and η have been found to be localized on endosomes (ref 27 and M.K.T. unpublished data). Again, little is known of the precise functional roles of these DGKs in each organelle, but some specific cases are discussed below.

1.6. Specific Interactions of DGKs with Other Proteins

As noted above, each DGK isotype is expressed in numerous tissues, and multiple DGK isotypes are often expressed in the same tissue and even within the same cell. Their overlapping expression patterns suggest that each DGK might have a specific set of biological functions. Evidence indicates that DGKs achieve specificity of function through their ability to regulate discrete pools of DAG. This occurs when DGK isoforms translocate to specific cellular compartments where they metabolize DAG. For example, DGKζ was shown to mediate DAG signaling downstream of the M1 muscarinic receptor (M1R), a seven-transmembrane receptor (GPCR).[11,41] Its translocation to M1R required binding to β-arrestins, which are scaffolding proteins that bind GPCRs. Blocking the interaction of β-arrestins with DGKζ attenuated DAG metabolism. The binding site on DGKζ for β-arrestins mapped to the C1 domains,[11] and mutating either of the C1 domains abolished translocation of DGKζ.[11,41] Since β-arrestins bind other GPCRs, this mechanism is likely broadly applied to limit DAG signaling initiated by many different GPCR agonists. Indeed, overexpressing DGKζ enhanced decay of ERK phosphorylation following activation of the gonadotropin-releasing hormone receptor, another GPCR.[48] In addition to influencing DAG signaling, DGK isotypes also generate PA, which can affect numerous proteins. This mechanism is discussed in more detail in section 5.1.

In addition to achieving specificity of function that is dictated by their intracellular localization (Figure 3), a second level of specificity occurs when DGKs bind to a unique subset of DAG- or PA-activated proteins in order to regulate their activity. This concept agrees with an emerging body of evidence indicating that specificity in signal transduction is often achieved by gathering together signaling proteins in common pathways along with their regulators.[49] Based on these two mechanisms that allow DGKs to achieve functional specificity, the outcome of DGK activity depends on the binding partners of each DGK isoform and the location in the cell where they bind these proteins. The effects that individual DGKs exert can be quite different. A clear example of this concept has been demonstrated for the type IV DGKs, ζ and ι, which are structurally very similar but have opposing effects on Ras signaling. DGKζ was found to attenuate Ras signaling, both *in vitro*[50,51] and *in vivo*,[51] while DGKι, which is structurally similar to DGKζ, enhanced Ras signaling.[52] These opposing effects on Ras are due to the ability of these DGKs to bind and inhibit specific RasGRP enzymes, which are DAG activated guanyl nucleotide exchange factors for Ras and Rap proteins. When DAG is abundant, RasGRP proteins activate Ras, Rap, or both. Because RasGRPs differ in their ability to regulate Ras or Rap, the outcome of RasGRP regulation by a DGK depends on which RasGRP is affected. For example, by metabolizing DAG, DGKζ inhibits the activity of RasGRP1,[50] which predominantly affects Ras. Thus, when DGKζ was overexpressed, Ras activation was reduced because RasGRP1 was inhibited.[50] Conversely, in conditions of DGKζ deficiency, Ras activity was enhanced because RasGRP1 was abnormally active. Its ability to regulate RasGRP1 was unique among the five other DGK isotypes that were tested; even an alternatively spliced form of DGKζ did not inhibit RasGRP1.[50] DGKι on the other hand binds and predominantly inhibits RasGRP3,[52] which affects both Ras and Rap. In conditions of DGKι deficiency, RasGRP3 activity was augmented, which led to excessive activation of Rap. Conversely, Ras activity was not increased but instead was reduced, suggesting that DGKι predominantly affected the ability of RasGRP3 to activate Rap, which then interfered with and reduced Ras signaling. Collectively these observations indicate that DGKs achieve functional specificity based upon the company that they keep.

Additional examples of DGKs specifically binding to DAG target proteins to regulate their activity have been published. For example, DGKα was also shown to bind and inhibit RasGRP1,[53,54] and DGKζ can bind and regulate PKCα.[53,54] Thus, it appears that DGKs commonly associate with and modulate proteins that are regulated by DAG. And given their ability to generate PA, it is not surprising that DGKs also bind and modulate proteins whose activity is regulated by PA. For example, through their ability to produce PA, DGKs have been shown to regulate either directly or indirectly Rac1, mTOR, and phosphatidylinositol-4-phosphate 5-kinase enzymes. These examples are discussed in detail in section 5.1. Based on the structural diversity of the DGK family, it is likely that each DGK regulates a distinct set of DAG signaling proteins, a concept that is supported by mouse knockout studies showing that mice with targeted deletion of individual DGK isoforms have distinct phenotypes.[30,52,55−57]

1.7. Regulation of DGK Activity

Activation of DGKs is complex and usually requires translocation of the DGK to a membrane compartment where it can bind to appropriate cofactors or other proteins. Additional regulation of their activity occurs by post-translational modifications. This complexity allows tissue- or cell-specific regulation depending on the availability of cofactors and the type of stimulus that the cell receives.

DGKα demonstrates the complex regulation of DGKs. Upon stimulation of T cells, DGKα translocates to at least two membrane compartments depending upon the agonist used. Stimulation of T cells with IL-2, for example, causes DGKα to translocate from the cytosol to a perinuclear region,[15,58] while activation of the T cell receptor results in translocation to the plasma membrane.[15] Once it arrives at a membrane compartment, the activity of DGKα can be modified by the availability of several cofactors. Calcium binds to the EF hand structures and stimulates its DAG kinase activity, and lipids such as phosphatidylinositol 3-kinase lipid products, phosphatidylserine, and sphingosine also activate DGKα.[59,60] Finally, DGKα can be phosphorylated by several protein kinases, including PKC isoforms and Src. Although the consequences of these phosphorylations are not clear, evidence suggests that phosphorylation by Src enhances DAG kinase activity.[61] Thus, several events can modify the activity of DGKα, and combinations of them appear to fine-tune its activity depending upon the cellular context.

Like DGKα, other DGK isotypes appear to be regulated by access to DAG through membrane translocation and by the availability of various cofactors. Members of each DGK subfamily appear to be regulated similarly, although probably there are subtle differences between subfamily members owing to

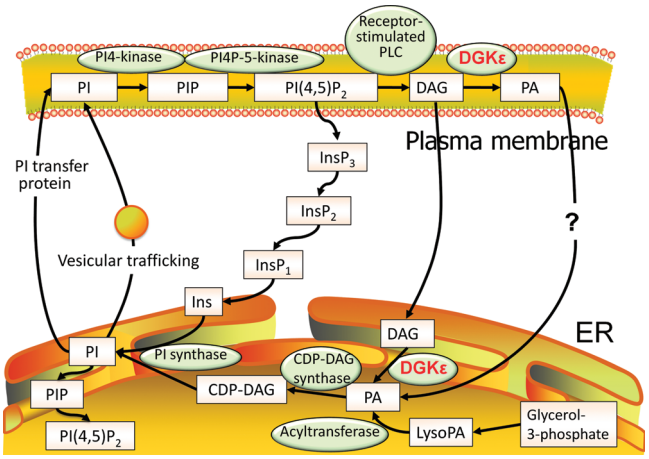

Figure 4. PI cycle between the plasma membrane and endoplasmic reticulum (ER).
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alternative splicing, tissue-specific expression patterns, unique binding partners, and subcellular localization. Similar to DGKα, the other type I DGKs are activated when they bind calcium via their EF hand motifs. Type II DGKs have a PH domain, and this motif in DGKδ binds to phosphatidylinositols, which do not activate DGKδ but might provide a localization cue.[13,19] Of note, conventional PKC isoforms phosphorylate serines within the PH domain, and this phosphorylation inhibits translocation of full length DGKδ1 or its isolated PH domain to the membrane.[62] Type II DGKs may also be negatively regulated through their SAM domains.[27] The activity of types III and IV DGKs can be modified by lipids: DGKε is inhibited by phosphatidylinositols and by phosphatidylserine, while type IV DGKs are activated by these anionic phospholipids.[63] The activity of type IV DGKs is also strongly regulated by their ability to access DAG in specific subcellular compartments. Both DGKs ζ and ι are imported into the nucleus, which requires their MARCKS homology domain that acts as a nuclear localization signal.[17,34] Nuclear import of DGKζ is regulated by conventional PKCs, which phosphorylate the MARCKS domain to inhibit this process.[17] Another level of nuclear import regulation appears to occur when the syntrophin family of scaffolding proteins binds to the carboxy-terminal PDZ binding domain of DGKζ. Binding to syntrophins sequesters DGKζ in the cytoplasm.[64] A third level of regulation occurs through a nuclear export signal in DGKζ.[65] Thus, nuclear accumulation of type IV DGKs is exquisitely regulated, suggesting an important nuclear function for these isozymes. Finally, DGKθ, a type V DGK, is inhibited when it associates with active RhoA,[7] and it translocates to the plasma membrane when PKCs ε and η phosphorylate it.[42]

1.8. Physiological Roles

Given the number of mammalian DGKs, their structural complexity, and their extensive tissue distribution, it is not surprising that these enzymes contribute to numerous biological processes. For example, based on their abundance in leukocytes and on inhibitor studies, DGKs have long been known to modulate leukocyte functions, but the DGK isoforms responsible for these effects were not known. Recently, however, studies in knockout mice have demonstrated that DGKs α and ζ suppress immune cell activation through the T cell receptor.[55,56,66,67] DGKζ also appears to function in mast cells downstream of the immunoglobulin E receptor and in macrophages downstream of Toll-like receptors.[68,69] Several DGKs have also been shown to modulate signaling events downstream of other plasma membrane receptors in a variety of cell types. For example, DGKs δ and η are required for proper EGFR signaling,[30,70] and DGKα acts downstream of the HGF and VEGF receptors.[71,72] In addition, DGKs help regulate small G protein signaling. For example, DGKs ι and ζ regulate Ras and Rap signaling by virtue of their ability to bind and regulate RasGRP exchange factors,[50,52] and DGKs γ and ζ help modulate the activation state of Rac1, albeit in opposing ways.[12,73] Finally, DGKε was found to be necessary for the maintenance of several signaling lipids in neuronal cells, and its deletion in mice protected them from induced seizures.[57] Collectively, these studies underscore the importance of DGKs in lipid signaling events and demonstrate the diversity of their functions. Many of these functions are discussed in more detail below.

2. FUNCTIONAL ROLES OF DGKS AT THE MOLECULAR AND BIOCHEMICAL LEVEL

2.1. Down Regulation of Diacylglycerol (DAG) and Up Regulation of Phosphatidic Acid (PA)

DGKs play a major role in cellular signaling by converting DAG to PA, regulating the balance between these two important lipid signaling molecules. Further modulation of this balance can be achieved by formation of DGK complexes with proteins that regulate DAG production or act as downstream effectors of DAG or with proteins that act downstream of PA to efficiently couple with the PA-dependent signaling.[3,74,75]

DAG is well-known to regulate different cellular processes, mostly through binding to C1 domains that are found in many proteins including protein kinase C (PKC).[76,77] In addition to modulating classical and novel DAG-sensitive PKC isoforms by removing DAG, DGKs have also been found to negatively regulate several other signaling proteins, such as RasGRP1,[50] RasGRP3,[52] UNC-13,[78,79] β_2-chimaerin,[80] and protein kinase D.[81] Also it was found that DAG activates some transient receptor potential channels that do not have C1 domains.[82]

The product of the reaction catalyzed by DGK, PA, has also been shown to regulate a wide variety of cellular events, including cytoskeletal rearrangement, proliferation, and cell survival.[83] It is also required for vesicle trafficking and stimulation of DNA synthesis and is potentially mitogenic. These effects are likely due to the ability of PA to regulate a number of signaling proteins, such as Sos, a Ras GEF (guanidine-nucleotide-exchange factor), Ras-GAP, phosphatidylinositol 5-kinases, Raf-1, PAK1, and PKCζ.

It is believed that each PA species, saturated and unsaturated, can differentially activate proteins. PA produced by DGKs is enriched in polyunsaturated fatty acids, particularly arachidonate. It was shown that the PA produced by DGKα was necessary for stimulated T lymphocytes to progress to S phase of the cell cycle[35] and that PA produced by DGKζ activates PAK1, which then causes actin rearrangements.[47]

The conversion of DAG to PA, catalyzed by DGKs, is also the first step in the resynthesis of phosphatidylinositols (PI). Therefore DGKs are crucial components of the PI cycle (see section 2.2).

2.2. Role of DGK in the PI Cycle

Several lines of evidence indicate that among all of the mammalian DGK isoforms, DGKε is uniquely involved in the PI cycle. All of the lipid intermediates in the PI cycle are enriched in arachidonoyl groups in the *sn*-2 position. The only DGK isoform that shows substrate specificity *in vitro* for DAG with an arachidonoyl acyl chain at the *sn*-2 position is DGKε.[84,85] Both the substrate and product of the reaction catalyzed by DGK, DAG and PA, are intermediates in the PI cycle (Figure 4). Hence the DGK-catalyzed step is a fundamental step in the PI cycle. Not only is there enrichment of arachidonic acid at the *sn*-2 position, but there is also the enrichment of *sn*-1 acyl chain of PI cycle intermediates with stearoyl. Thus, the most common acyl chain composition of lipid intermediates in the PI cycle is 1-stearoyl-2-arachidonoyl, and the DAG substrate with this acyl chain composition is phosphorylated at the highest rate compared with other molecular species of DAG.[86] This same acyl chain preference is also exhibited in the competitive inhibition of DGKε by phosphatidic acid.[86] No other isoform of DGK exhibits any acyl chain specificity. Recently lipidomics analysis has been carried out to compare the acyl chain composition of the major phospholipids in normally proliferating mouse embryonic fibroblasts (MEFs) derived from wild-type versus DGKε or DGKα knockout mice.[87] Dramatic differences between wild-type and DGKε knockout cells in arachidonate-containing lipids were observed for multiple classes of glycerophospholipids and polyphosphatidylinositides. The peaks from mass spectrometry are identified by their mass/charge ratio and presented in the form *X*:*Y* where *X* is the total number of carbon atoms and *Y* the total number of double bonds in both acyl chains of the phospholipid. The lipidomics data demonstrated that the 38:4 species of PI deceased from 33% of the total cellular PIs in the wild-type cells to 24% in the DGKε knockout cells, a dramatic decrease of 27%.[87] Further, 18:0 (stearoyl)-containing PI decreased by 29% in DGKε knockout cells compared with the wild-type samples ($p < 0.01$).[86] This is in contrast to the observation that despite the similarity between 16:0 and 18:0 acyl chains, deletion of DGKε results in a larger decrease in 18:0 compared with 16:0 PI, supporting DGKε's selectivity for an *sn*-1 stearoyl acyl chain of DAG[86] in addition to the arachidonoyl specificity for the *sn*-2 position. These results provide *in vivo* evidence of DGKε's selectivity for DAG with a 1-stearoyl-2-arachidonoyl acyl chain composition resulting in the enrichment of PI with this acyl chain composition. Hence, DGKε can affect PIs that are neither substrates nor products of the DGK reaction but are influenced by DGKε through the PI cycle.

In contrast to the observations with DGKε, no differences in the acyl chain composition of any phospholipid class or DAG were observed between wild-type and DGKα knockout cells. There was also no significant difference in the concentrations of any of the DAG species between the wild-type and DGKε knockout MEFs. However, the cells from the DGKα knockout mice had a higher concentration of DAG, consistent with the lack of down regulation of the major fraction of DAG because of the absence of DGKα. This is in contrast with DGKε that is primarily responsible for enrichment of only a fraction of PIP_n, that is, species with arachidonoyl acyl chains.

One of the proposed roles of DGK in regulating metabolism has been suggested to be the removal of the signaling lipid DAG. The results with the DGKα knockout MEFs are in accord with this explanation, since removal of this isoform results in a slower loss of DAG and hence an increase in its concentration. We anticipate that this effect is typical of most, if not all, mammalian isoforms of DGK, apart from DGKε, because these isoforms phosphorylate all forms of DAG with equal rates. This is not the case, however, for DGKε, which has specificity for catalyzing the phosphorylation of SAG. Hence there is less effect on the total concentration of DAG since SAG appears to be the principle form of DAG that is phosphorylated by DGKε and the other species of DAG should not change their concentration.

However, the unique enzyme specificity of DGKε does not explain certain other observations. The degree of specificity of DGKε for SAG compared with other structurally related forms of DAG with small differences in acyl chain length or degree of unsaturation is relatively modest. However, the enrichment of PI cycle intermediates with SA forms of the lipids is substantial. This can be explained by the fact that the PI cycle is a catalytic cycle in which the intermediates are regenerated each time the cycle goes around. As a consequence, there is a progressive enrichment of SA forms of the lipid intermediates as a consequence of the cyclical nature of this metabolic pathway. In addition, although, as stated above, most species of DAG increase to a similar extent in DGKα knockout MEFs, the 38:4 species and closely related DAG species are exceptions that remain unchanged in DGKα knockout cells.[87] The principle component of this DAG would be the species SAG, an intermediate in PI cycling. One explanation for the lack of increase of 38:4 DAG when all of the other species of DAG increase in DGKα knockout MEFs is that there is compartmentalization in the cell so that SAG is in a compartment that contains DGKε but not DGKα. Hence the deletion of DGKα has no effect on this species of DAG because the substrate is not physically accessible to the enzyme. However, this does not explain why there is no change in 38:4 DAG in DGKε knockout MEF.[87] We also observe that there is a decrease in the concentration of all of the lipid intermediates of the PI cycle in the DGKε knockout cells.[88] Perhaps two things counterbalance each other, that is, an increase in 38:4 DAG as a result of lowering the conversion of SAG to SAPA as a consequence of knocking out DGKε and a decrease in 38:4 DAG as part of an overall decrease in PI cycle lipid intermediates. The reason for the lowing of the concentrations of PI cycle intermediates is that when the PI cycle is slowed the intermediates in the PI cycle will be metabolized by alternative routes to other products. Inhibiting any step in the PI cycle will slow all the steps in the cycle, because the PI cycle is a catalytic cycle in which intermediates are regenerated each time the cycle goes around. There is also a feed-forward mechanism within the PI cycle in which the PA generated by DGK activates phosphatidylinositol-4-phosphate 5-kinase (PI4P5K), which catalyzes the conversion of PI4P to PIP_2, one of the steps in the PI cycle. It should be noted, however, that SAPA is not a potent activator of human type Iα PI4P5K but rather PAs with dipolyunsaturated acyl chains are found to be the best activators.[89] Hence there are two mechanisms by which knocking out DGKε will slow the PI cycle, one is directly by removing the enzyme that catalyzes one of the steps in the PI cycle, that is, the conversion of SAG to SAPA, and the other mechanism is through the lowering the concentration of SAPA, an intermediate in the PI cycle, resulting in a lowering of all the PI cycle intermediates. Although PA can also be formed through the action of phospholipase D, the path through DGK appears to be the more important one for cell regulation.[90]

Another observation, that goes counter to the hypothesis that the sole function of DGK is to down-regulate the DAG signal, comes from observations that electrical stimulation of the brains

of mice leads to a transient increase in arachidonoyl-containing DAG.[57] Interestingly, this increase is greater in wild-type mice compared with DGKε knockout mice. Again this observation is contrary to the concept of DGK lowering the level of DAG. However, the observation would be consistent with the idea that removing DGKε slows the PI cycle and hence results in the lowering of all the intermediates in the cycle.

We can thus conclude that whatever the details of the regulation of lipid intermediates of the PI cycle, it is clear that DGKε, but not other isoforms of DGK, is the predominant enzyme that catalyzes the step of the PI cycle in which SAG is phosphorylated to SAPA.

3. ROLE OF DGK AT THE CELLULAR LEVEL

3.1. Cell Division and Proliferation

DGKs have been shown to modulate cell cycle dynamics by several different mechanisms. As noted above, several DGK isoforms are found within the nucleus where they are thought to regulate DAG or PA signaling that occurs as part of the nuclear phosphatidylinositol cycle.[91] DAG is present in nuclear preparations and appears to fluctuate with the cell cycle, but the specific pattern of its accumulation is not clear because of the many different methods used to isolate nuclei. However, most data suggest that nuclear DAG peaks shortly before S phase and promotes the G1/S transition,[92] facilitating cell cycle progression. Consistent with this and emphasizing the importance of nuclear DAG signaling in the cell cycle, cells overexpressing DGKζ were inhibited from progressing to S phase, presumably because DGK reduced the levels of nuclear DAG.[17] A kinase inactive mutant DGKζ did not affect the cell cycle, further supporting a model in which DGKζ modulates the cell cycle by metabolizing nuclear DAG.[17] The targets of nuclear DAG are not clear at this point, and it is still somewhat of a mystery exactly where within the nucleus DAG and other signaling lipids exist. However, one clue to the function of nuclear DGKζ and DAG might be that DGKζ localizes with chromatin within the nucleus[93] and also binds, through its MARCKS homology domain, the retinoblastoma protein (pRB)[94] the best-known function of which is to repress transcription of E2F-regulated genes that are needed for cell proliferation. Binding of pRb augments the activity of DGKζ and their association is inhibited when protein kinase C phosphorylates the MARCKS homology domain in DGKζ.[95] The retinoblastoma protein also binds other lipid kinases and may act as a scaffold protein to integrate nuclear phosphatidylinositol signaling. This complex of proteins could provide a link between changes in nuclear signaling lipids and cell cycle regulation.

DGKs α, γ, ι, and θ have also been observed in nuclei,[96] and some are confined to specific compartments within the nucleus. This compartmentalization suggests that DGK isotypes have specific roles in the nucleus. Their roles within the nucleus remain largely unexplored, but there is evidence that DGKs can have both positive and negative effects on the cell cycle. For example in T lymphocytes, the PA produced by nuclear DGKα appears to be necessary for IL-2-mediated progression to S phase of the cell cycle,[35] and expression of a kinase-deficient mutant DGKγ reduced cell cycle progression.[96] Collectively, these data indicate both the complexity and importance of lipid signaling and DGK function in the nucleus, but it is clear that further work needs to be done to completely understand their nuclear roles.

In addition to affecting cell cycle progression by modulating lipid signaling events within the nucleus, DGKs also regulate cell proliferation from outside of the nucleus. They accomplish this task largely by modulating signaling cascades that promote cell cycle progression. For example, both DGKδ and DGKη are necessary for proper cell proliferation by virtue of their regulation of EGFR signaling,[30,70] and DGKα is required for cell proliferation mediated by a constitutively active anaplastic lymphoma kinase fusion protein.[97] These DGKs modulate proliferation by different mechanisms, and in the case of DGKη, it appears that its activity is not required to modulate cell proliferation. The roles of these DGKs in cell proliferation and cancer are discussed in more detail in section 4.2.

3.2. Cell Morphology: Cytoskeletal Rearrangements

It was found that some DGK isoforms are localized to the cytoskeleton, suggesting their role in regulating cytoskeleton rearrangements. Thus, it was shown that DGKα is required for growth-factor-induced cell migration and ruffling, through activation of Rac small-GTPase and coordination of its localization.[98] Recently, the details of this signaling pathway were unveiled. Chianale et al.[71] showed that upon hepatocyte growth-factor stimulation, DGKα, through production of PA, regulates the recruitment of atypical PKCζ/ι in complex with RhoGDI and Rac to ruffling sites of epithelial cells. In turn, activation of atypical PKCζ/ι promotes the release of Rac from the inhibitory complex with RhoGDI, leading to its activation and, consequently, to formation of membrane ruffles. Another isoform, DGKγ, was shown to colocalize with F-actin upon phorbol ester treatment.[76] A number of observations suggest that DGKγ acts as an upstream suppressor of Rac1 and, consequently, lamellipodium/membrane ruffle formation. Thus, it was proposed that DGKγ might affect cytoskeleton dynamics through regulating Rac1 activity during leukemia cell differentiation.[99] It is also possible that by affecting Rac1 activity, DGKγ indirectly regulates gene expression of proteins responsible for the differentiation.[100] Additionally, it was found that in response to epidermal growth factor cell stimulation, DGKγ activates β2-chimaerin, a Rac-specific GAP, most likely through PA produced by its catalytic activity, thus suggesting that β2-chimaerin is an effector molecule that functionally links DGKγ with Rac1.[80]

Recently it was found that the epsilon isoform of DGK is colocalized with actin stress fibers in isolated rat aortic smooth muscle cells.[101] It was shown that upon serotonin-stimulated contraction of the cells, DGKε was detected diffusely in the cytoplasm without a filamentous stress fiber pattern. Moreover, inhibition of Rho-associated kinases by Y-27632 or inhibition of actin polymerization by cytochalasin B lead to a decrease in the intensity of DGKε signal on stress fibers, thus, suggesting that DGKε interacts with actin stress fibers and may be involved in their stability in vascular smooth muscle cells.

DGKζ was reported to interact with the cytoskeleton near the plasma membrane at the leading edge of migrating cells.[12] This interaction occurs through DGKζ−syntrophin complex, which is translocated from the cytosol to the plasma membrane upon PKC-dependent phosphorylation of the DGKζ−MARCKS−PSD in skeletal muscle.[102] It was shown that in N1E-115 neuroblastoma cells a complex formed by DGKζ, syntrophin, and Rac1 controls neurite outgrowth.[12] And recently it was demonstrated that DGKζ-deficient fibroblasts were defective in Rac1-regulated processes, such as lamellipodia formation,

dx.doi.org/10.1021/cr1004106 |Chem. Rev. 2011, 111, 6186–6208

membrane ruffling, migration, and spreading.[47] The results of this study suggest that DGKζ forms a stable complex with PAK1 and RhoGDI that acts as a Rac1-selective RhoGDI dissociation factor. Thus, DGKζ through generating PA activates PAK1, which leads to the dissociation of RhoGDI from Rac1, causing changes in cytoskeleton dynamics.

3.3. Movement of Lipid among Organelles

Different isoforms of DGK are found in different locations within the cell.[45] We have discussed herein (section 1.5) current knowledge about the subcellular localization of the various isoforms of DGK. Their specific location will contribute to a depletion of DAG and a build up of PA in the organelle in which a particular isoform is located. To maintain homeostasis there would have to be a compensatory movement of lipids among organelles or stimulation of other metabolic steps.

There is one metabolic pathway for which lipid movement between organelles plays a critical role. This is PI cycling in which DGK, particularly DGKε, catalyzes one of the steps. This cycle involves steps that occur only in the endoplasmic reticulum (ER), while the stimulation of other steps occurs in the plasma membrane. There are also reactions that take place in both membranes. Nevertheless, PI is synthesized only in the ER while the formation and cleavage of PIP_2 occurs mainly in the plasma membrane, although there are enzymes in the ER that can phosphorylate PI.[103] It is possible that there is a limited amount of PI cycling within the ER that does not require lipid transfer. However, in order for the PI cycle to be efficient and sensitive to hormone stimulation, there must be transfer of PI from the ER to the plasma membrane and counterbalancing transfer of lipid, in the form of DAG, PA, or both, from the plasma membrane to the ER.

PI formed in the ER can be transferred to the plasma membrane by both specific lipid transporters[104,105] and vesicular transport. There is also recent evidence (T. Balla, personal communication) that PI can be formed in small particles that are present outside of both the ER and the plasma membrane. These particles carry PI synthetase and may be responsible for delivering PI to other membranes of the cell. It has been shown that PA is required for vesicular trafficking. PA regulates fusion through promotion of negative membrane curvature.[83] Moreover, PIP_2 also regulates vesicular transport.[106] Hence the rate of the vesicular transport component of movement of PI from the ER to the plasma membrane is subject to modulation by the concentration of certain intermediates of the PI cycle. If the PI cycle is inhibited, as for example by the removal of DGKε, then the concentration of PI cycle lipid intermediates will decrease resulting in the further slowing of the cycle because of loss of lipid factors that facilitate vesicular transport of lipids from the ER to the plasma membrane.

Less is known about the transfer of lipid from the plasma membrane to the ER. It is known that there are contact sites where there is close juxtaposition of the ER and plasma membranes. It is at these sites where lipid transfer may be facilitated.[107−110] With regard to the PI cycle, there are two lipids that could be transferred in this direction to complete the cycle, either DAG or PA. DGKε is present in both the ER and the plasma membrane.[45,111] Hence the SAG formed from the PIP_2-specific phospholipase C in the plasma membrane can either be transferred to the ER where it would be converted to SAPA by the DGKε in the ER or be converted to SAPA in the plasma membrane, also by DGKε, and then the SAPA would be transferred to the ER. Little information is available with regard to which of these two pathways has greater importance for completing the PI cycle. It is known that PA transfer is not dependent on soluble lipid carriers or on vesicular transport[112] as was the case of PI transfer to the plasma membrane. In addition to transfer from the plasma membrane, there is an alternative pathway for the *de novo* synthesis of PA from glycerol-3-phosphate directly in the ER.[113] Using acyl-CoA's, PA is first synthesized and undergoes maturation in the remodeling pathway that includes acylation of lyso-PA (Lands' cycle).[114] This newly synthesized PA can then enter the PI cycle in the ER through a CDP-dependent reaction catalyzed by CDP-diacylglycerol synthase. CDP-diacylglycerol synthase is not found in the plasma membrane, nor can the plasma membrane synthesize PA from small molecule precursors. However, the acyl chain composition is very similar for PI or PA in the ER vs the plasma membrane, but the acyl chain profile for PI is very different from that for PA.[88] The reason the acyl chain composition of these two lipid classes are different is that there are other major sources of PA, independent of the PI cycle. The other major source of PA is as a product of reactions catalyzed by phospholipase D. Since there are different lipid metabolizing enzymes in the two membranes, the ER and the plasma membrane, the similar acyl chain composition of PI and of PA in the two membranes provides good evidence for the facile exchange of these two lipid species between the two membranes.

3.4. Formation of lipid droplets

In addition to its function as a lipid signal, DAG is also a key intermediate in lipid metabolism.[115] It is the substrate for the synthesis of the phospholipids PC, PE, and PS, and through its conversion to PA by DGK, DAG provides the precursor, PA, for the synthesis of PI, phosphatidylglycerol, and cardiolipin. In addition, SAG can be acted upon by a phospholipase A1 to produce 2-arachidonoyl glycerol, an important ligand for the CB1 cannabinoid receptor.[116] In addition, DAG can be acted upon by acyl-CoA:diacylglycerol acyltransferase to form triglycerides.

DAG is not only a precursor for the formation of fats for lipid storage, but it is also the trigger for the formation of lipid droplets through the recruitment of proteins, such as perilipin, that facilitate lipid droplet formation.[117] Lipid droplets can be formed from caveolae in some cells, but more generally, lipid droplets arise in the ER as a result of the accumulation of DAG between the phospholipid leaflets.[118,119] Thus in addition to altering the concentration of the lipid signaling molecules, DAG and PA, DGK also inhibits the formation of lipid droplets both by removing DAG, which both removes the immediate substrate for the formation of triglycerides, as well as removing the lipid signal that triggers the formation of the lipid droplet structure.

4. RELATIONSHIPS OF MOLECULAR AND CELLULAR PROPERTIES TO PHYSIOLOGICAL FUNCTION

4.1. Regulation of Immune Function

Based on inhibitor studies, DGKs have long been known to modulate leukocyte functions, but the specific DGKs responsible for these effects were not known. Recently, however, studies in knockout mice have demonstrated important roles for DGKs α and ζ in white blood cells. For example, lymphocytes from DGKζ knockout mice had excessive Ras signaling following T

cell receptor stimulation, and consequently, they were hyperproliferative.[55] The abnormal Ras signaling in the DGKζ-deficient lymphocytes was probably due to high levels of DAG that then activated RasGRP1.[50] DGKζ also appears to function downstream of the immunoglobulin E receptor in mast cells and downstream of Toll-like receptors in macrophages.[68,69] But unlike its role downstream of the T cell receptor, its function in these cells might be to produce PA rather than to metabolize DAG.

Similar to DGKζ, DGKα also functions downstream of the T cell receptor, and deleting its gene in mice or treating lymphocytes with inhibitors of DGKα enhanced T cell receptor signaling and promoted proliferation.[56,66] Ultimately, DGKα deficiency caused T lymphocytes to become resistant to anergy, a state of tolerance to antigens. Conversely, overexpressing DGKα promoted anergy. Like DGKζ, DGKα appears to modulate DAG and RasGRP1 signaling downstream of the T cell receptor so that in conditions of DGKα deficiency, DAG levels rise, RasGRP1 is activated, and Ras signaling is abnormally high. Consistent with this model in which a DGK modulates lymphocyte tolerance by regulating DAG and RasGRP1 signaling, deleting DGKζ, which is known to regulate RasGRP1, similarly modulated T cell anergy.[56] Together, these data indicate that DGKs α and ζ regulate lymphocyte tolerance through their ability to regulate DAG signaling downstream of the T cell receptor. Since both of these DGKs appeared to function similarly in lymphocytes, it was not surprising to find that lymphocytes in which both DGKα and DGKζ had been deleted had severe defects in T cell maturation, and a small portion of the double knockout mice developed thymic lymphomas when they expressed a transgene that promoted thymic tumorigenesis.[67] Collectively, these data indicate that DGKs α and ζ have prominent and synergistic roles in regulating T lymphocyte signaling. In addition to DGKα and DGKζ, other DGK isoforms are expressed in leukocytes, but their specific function *in vivo* remains to be established.

4.2. Cell Proliferation and Cancer

DAG has long been known to facilitate cell transformation. For example, cell lines that overexpress PLCγ, which causes excess DAG, have a malignant phenotype.[120] Conversely, cells manipulated to reduce the expression of PLCs β, γ, or δ have a suppressed growth response.[121] Additionally, there are elevated DAG levels in cells overexpressing Ras and other oncogenes.[122−125] Although implicating the transforming potential of excess diacylglycerol, these studies did not address a direct role for DAG in this process. More direct evidence that abnormally high DAG levels are involved in cellular transformation comes from studies using phorbol esters. This complex family of compounds promotes tumorigenesis by acting as DAG analogues that are metabolized very slowly. This results in prolonged activation of proteins that would normally bind DAG only briefly. Based on the tumor-promoting properties of phorbol esters, it is likely that abnormally elevated DAG levels caused by its dysregulated metabolism would have similar tumor promoting effects. As such, one would predict that reduced expression or activity of DGKs would lead to excessive DAG levels that could promote cell transformation.

Probably the best example of a DGK that negatively regulates DAG and consequently limits the transforming potential of this lipid is DGKζ, which functions both inside and outside of the nucleus to achieve its effects. Within the nucleus, DGKζ limits DAG levels and consequently alters cell cycle dynamics, and as expected, overabundance of DGKζ slowed the cell cycle.[17] Outside of the nucleus, DGKζ regulates DAG signaling

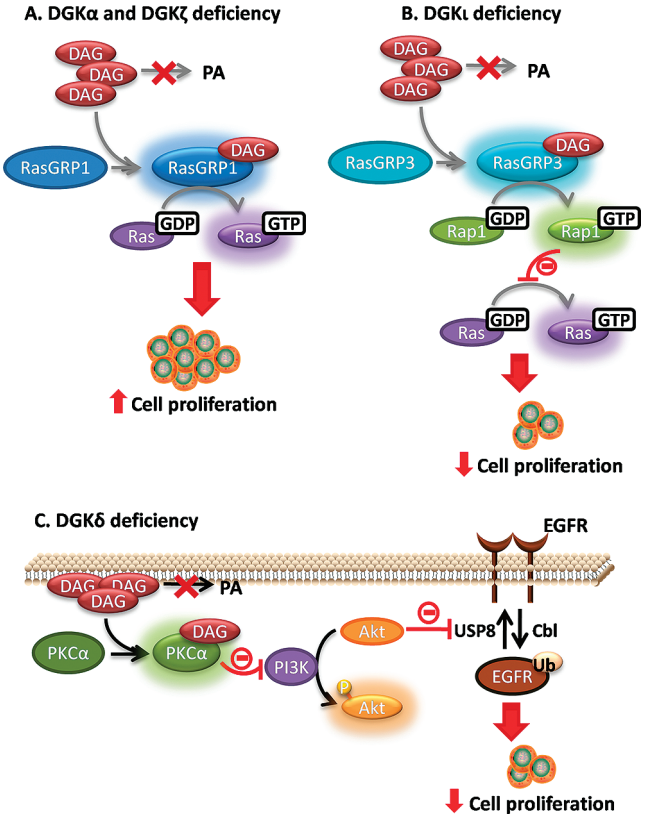

Figure 5. Role of DGKs in cancer and cell proliferation. (A) DGKα or DGKζ deficiency results in increased cell proliferation and more tumors. (B) DGKι deficiency results in decreased cell proliferation and fewer tumors. (C) DGKδ deficiency leads to significantly reduced levels and activity of the EGFR, resulting in decreased cell proliferation. PI3K, phosphatidylinositol 3-kinase; Cbl, E3 ubiquitin ligase; EGFR, epidermal growth factor receptor; USP8, ubiquitin-specific protease 8; EGFR-Ub, ubiquitinated EGFR.

downstream of the T cell receptor to affect RasGRP1, an enzyme that helps promote proliferation following engagement of the T cell receptor. As expected of a negative regulator of RasGRP1, DGKζ-deficient lymphocytes had excessive Ras activation and were hyperproliferative following activation of the T cell receptor. DGKα also appears to modulate RasGRP1 and its deletion also led to hyperproliferation of T cells[68] (Figure 5A). These results lent credence to the possibility that deleting or inhibiting a DGK would universally promote cell transformation as a consequence of excessive DAG signaling. However, that has not been the case: DGK deficiency reduces the transformation potential of cells at least as often as it promotes transformation. For example, disruption of the DGKι gene in mice led to significantly fewer skin tumors.[52] These effects were in contrast to those of disrupting the DGKζ gene, which led to a trend toward more tumors. These differences between DGKι and DGKζ were caused by selective regulation of different RasGRP enzymes: deletion of DGKι promoted the activity of RasGRP3, which activated Rap1, leading to reduced Ras activity,[52] while deleting DGKζ promoted the activity of RasGRP1, which led to excessive Ras activity[50] (Figure 5B). Thus, it appears that even though DGKs negatively regulate DAG signaling, the effects of manipulating their function depend on the targets that are downstream of that specific DGK isoform.

dx.doi.org/10.1021/cr1004106 |*Chem. Rev.* 2011, 111, 6186–6208

Another example of a DGK whose deletion reduces the transformation potential of a cell is DGKδ. Cells in which the gene encoding DGKδ had been deleted had abnormally high DAG levels.[30] But despite the excessive DAG, DGKδ-deficient mice had a phenotype very similar to epidermal growth factor receptor (EGFR) null mice.[30] The EGFR is a prominent cancer target that is highly expressed or excessively active in many cancers. In DGKδ-deficient cells and tissues, the levels and activity of the EGFR were significantly reduced. These defects in EGFR signaling were due, in part, to excessive PKC activity that led to reduced activation of EGFR, promoted its degradation, and reduced the proliferation of cells[30,126] (Figure 5C). Thus, even though DGKδ deficiency led to excessive DAG levels, this did not result in transformation, but instead it reduced proliferation. Similar to DGKδ, another type II DGK, DGKη, also appears to modulate EGFR signaling. Like DGKδ, when the levels of DGKη were reduced, cell proliferation was inhibited,[70] offering additional evidence that DGKs do not universally inhibit cell growth. The effects of DGKη on signaling downstream of the EGFR appeared to be independent of its DGK activity.

Finally, some DGK isoforms can have opposing effects on cell proliferation. As noted above, deletion of DGKα in lymphocytes led to their hyperproliferation following engagement of the T cell receptor. Thus, downstream of the T cell receptor, DGKα acts to limit cell proliferation. The effects of deleting DGKα in this context are likely due, in part, to accumulation of DAG and consequent activation of RasGRP1.[50] In contrast to its effects downstream of the T cell receptor, in lymphocytes treated with interleukin-2, DGKα promoted cell cycle progression,[58] and in lymphoma cells expressing an activated form of the anaplastic lymphoma kinase receptor, DGKα also promoted proliferation.[97] These contrasting effects of DGKα were probably due to its subcellular localization or the targets of DAG or PA that DGKα modulates. They serve as an example of the diverse roles that DGKs have in the process of cell proliferation and tumorigenesis.

4.3. Brain Function

Most of DGK isoforms are expressed abundantly in the brain and some of them in retina, suggesting the physiological importance of this protein in the central nervous system and visual function. In situ hybridization analysis showed that mRNA for each isoform is expressed in a distinct pattern in the brain.[127] Thus, DGKα is detected in glial cells in the white matter but not in the neurons,[127] and this enzyme was proposed to be an important component of myelin regeneration.[128]

Although DGKβ localization is not limited to the brain tissues, in rat brain, it is expressed specifically in those areas that are important in synaptic transmission of cognitive and emotional processes in the central nervous system.[129,130] It was also found that in the hippocampus DGKβ is detected not only in projection neurons but also in interneurons and is abundant at perisynaptic sites and that DGKβ induces dendrite outgrowth and spine maturation in developing hippocampal neurons through its enzymatic activity.[131] Shirai et al. showed further that membrane-localized DGKβ regulates the neurite spine formation and its loss results in lower spine density causing impairment of LTP in the hippocampal CA1 region and memory related to cognitive functions, including spatial and long-term memory.[132] Moreover, it was shown that localization pattern of DGKβ in the pituitary intermediate lobe is similar but not identical to that of dopamine 2-like receptor (D2R) and PLCβ4, suggesting that the D2R/PLCβ4−DGKβ cascade is likely to operate dopaminergic transmission in the intermediate lobe cells.[133] A recent study also demonstrated that DGKβ knockout mice showed psychomotor behavioral abnormalities, such as hyperactivity, reduced anxiety, and reduced depression, that were attenuated by the administration of the mood stabilizer lithium but not haloperidol, diazepam, or imipramine.[134] These lithium-sensitive behavioral abnormalities in DGKβ knockout mice are suggested to be due to the impairment of Akt-glycogen synthesis kinase 3β signaling and cortical spine formation.[134]

The DGKγ isoform is expressed abundantly in brain and retina, whereas most other tissues express an inactive truncated form of DGKγ.[25] It was suggested that there is a functional correlation between DGKγ and PKCγ through attenuation of PKCγ activity by DAG consumption.[115] PKCγ is predominantly localized in the postsynaptic dendrites in the hippocampal pyramidal cells, and the activation of postsynaptic PKC appears to be necessary for the induction of LTP.[135] Because DGKγ is also highly expressed in the same cells, it could suggest the involvement of DGKγ in LTP through negative regulation of PKCγ.

DGKε isoform is very abundant in brain and retina,[57] but it is more interesting that its brain distribution mirrors that of mGluR1 (G-protein-coupled glutamate metabolic receptor 1),[136] suggesting that DGKε regulates mGluR-depending signaling. Studies with DGKε-deficient mice showed that they are resistant to electroconvulsive shock with shorter tonic seizures and faster recovery than wild-type mice.[57] Moreover, hippocampal dentate gyrus neuronal cells from DGKε knockout mice showed resistance to long-term potentiation. Importantly, these responses are associated with impairment of the PI cycle through reduced PIP_2 degradation and reduced accumulation of free arachidonic acid, since conversion of arachidonoyl-DAG by DGKε is the first step in the resynthesis of PIs. The data also suggest that DGKε positively modulates kindling epileptogenesis[137] and that DGKε signaling is required for regulation of epilepsy-induced genes, such as COX-2 (cyclo-oxygenase 2) and tyrosine hydroxylase.[138]

DGKδ demonstrated a broad neuronal expression with high levels in pyramidal neurons in the neocortex and hippocampus and within internal granule cell neurons of the cerebellum.[139] In contrast to the effect caused by DGKε deficiency, it was reported that a patient lacking DGKδ2 isoform exhibited developmental delay and seizures, suggesting that up-regulation of this enzyme can be a potential drug target for epilepsy.[140]

DGKζ seems to be the isoform that is most abundantly expressed in the brain, with the highest levels in hippocampus, cerebral and cerebellar cortices, and olfactory bulb.[127] One of the important functions of DGKζ is a regulation of neurite formation by directing translocation of its complex with RacGDP and syntrophins to growth cones.[12]

It was also suggested that DGKζ efficiently regulates synaptic local DAG concentration for the maintenance of normal spine density and excitatory synaptic transmission.[141] DGKζ is recruited to excitatory synapses by PSD-95, which may target DGKζ close to the proteins that initiate DAG signaling (NMDA receptors and mGluRs) to tightly couple DAG production to its conversion to PA. Under normal conditions, DGKζ is shown to be expressed in neurons but not in glial cells.[142] However, it was found that under pathological conditions, such as cerebral ischemia or infarction, DGKζ quickly disappears from ischemic neurons but later appears in glial cells,[143] suggesting a role for DGKζ in glial functions under pathological conditions. Further it

was demonstrated with the brain cryoinjury model that DGKζ is induced in activated microglia in the course of glial scar formation.[144]

Another suggested function of DGKζ in brain is a control of body fat accumulation by regulating DAG levels in the leptin signaling pathway. Leptin regulates body weight through activation of the Ob−Rb receptor, and mutations in this hormone or its receptor decrease DGKζ activity in the hypothalamus and cause abnormal obesity.[145]

DGK*η* was shown to be expressed dominantly in the brain,[130] and recently this isoform was linked to the pathogenesis of bipolar disorder.[146] It was demonstrated that the association of the *DGKH* gene with bipolar disorder was replicated at the haplotype level in a Sardinian sample,[147] but it was not confirmed for individual SNPs.[148] In another study, it was shown that *DGKH* gene expression levels in individuals with bipolar disorder were 25% higher than in controls, although the bipolar disorder group did not differ significantly from the schizophrenia group, bringing the possibility that this increase is not specific for the bipolar disorder.[149]

DGK*θ* is detected ubiquitously in the gray matter region, similar to DGKε distribution, suggesting a general role in neuronal functions.[150] The studies with *Caenorhabditis elegans* provide evidence that DGK*θ*, by regulating local DAG levels, may participate in the regulation of synaptic transmission. Thus, ablation of DGK-1, a DGK*θ* orthologue, in *C. elegans* led to an increase in synaptic transmission, as well as defects in feeding behavior, locomotion, and egg laying.[79]

In addition to synaptic transmission, DGK*θ* may play an important role in gene transcription and regulation in neuronal cells.[151] Although nuclear localization of DGK*θ* in a variety of cell types was confirmed in several studies,[38,152] the physiological significance of the nuclear DGK*θ* has not yet been established. However, it was shown that by producing PA, DGK*θ* activates the nuclear receptor steroidogenic factor 1, a transcription factor responsible for activation of cytochrome P450 transcription and regulation of steroidogenesis.[153] Further, silencing of DGK*θ* expression, but not DGKζ, inhibits CYP17 transcription.

Together, these data suggest that DGKs have multiple functions in the regulation of the nervous system, yet much more needs to be understood about the specific roles and physiological significance of each isoform.

4.4. Cardiac Function

Based on Northern blot analysis, the two principle isoforms of DGK that are expressed in the heart are DGKε and DGKζ, along with a lower level of DGKα.[154] Interestingly, the two principle DGK isoforms of cardiac muscle appear to exhibit opposite changes in response to myocardial dysfunction. After myocardial infarction, levels of DGKζ are greater in the periphery of the necrotic tissue, which could be attributed to granulocytes and macrophages infiltrating into this area.[154] In contrast, the expression of DGKε was lower in the infarcted and border areas.[154] In a similar manner, cardiac hypertrophy affects DGKε and DGKζ in different ways. After aortic banding, expression of mRNA of DGKε significantly decreases but that of DGKζ does not.[155]

In contrast to the opposite changes in expression levels of DGKε and DGKζ in response to myocardial damage, both of these DGK isoforms have been shown to protect against cardiac dysfunction resulting from several causes. In the case of DGKε the increased heart weight and cardiac dysfunction observed in wild-type mice as a result of transverse aortic constriction was abolished in mice with cardiac-specific expression of DGKε.[156] It is known that the signal transduction pathway from a $G\alpha_q$ protein-coupled receptor (GPCR) plays a critical role in the development of cardiac hypertrophy and heart failure.[157,158] Stimulation of this signal transduction pathway with phenylephrine resulted in the accumulation of DAG and the translocation of PKC isoforms to membranes. These effects were blocked in the mice with cardiac-specific expression of DGKε.[156] In addition, the up regulation of the transient receptor potential channel as a result of transverse aortic constriction is attenuated in the mice with cardiac-specific expression of DGKε.[156] These results show that the cardiac expression of DGKε can reverse cardiac dysfunction and improve survival by controlling cellular DAG levels and the expression of the transient receptor potential channel.

Similar experiments were done with DGKζ, rather than DGKε, showing that increased expression of DGKζ also protected the heart from damage. Several studies have focused on this isoform in relation to the heart, in part because it is the predominant isoform in the rat myocardium.[154] It has been shown that overexpression of DGKζ in rat neonatal cardiomyocytes, as well as *in vivo* in mouse hearts, prevents over activation of PKC and improves the course of left ventricle remodeling in the infarcted myocardium,[159] in cardiac hypertrophy induced by angiotensin II or by phenylephrine,[160] and in pressure-induced cardiac hypertrophy,[161] as well as inhibiting myocardial atrophy and restoring cardiac function in mice with streptozotocin-induced diabetes mellitus.[162] In analogy with the inhibition of $G_{\alpha q}$-induced effects on the mouse heart by DGKε,[156] similar results were observed with the overexpression of DGKζ.[163,164]

Although in response to myocardial damage, the two principle cardiac isoforms of DGK change expression levels in opposite directions, overexpression of both DGKε and DGKζ in the heart results in protection against myocardial damage by a variety of mechanisms. Of course these two results are not necessarily contradictory because the two phenomena are independent of each other. Nevertheless, the similar effects with regard to cardiac protection is surprising because of the marked differences between these two isoforms with DGKζ being an amphitropic protein that can also translocate to the nucleus, while DGKε appears to be permanently membrane bound. Additionally, DGKε but not DGKζ exhibits specificity for DAG substrates with arachidonoyl groups. Each of the isoforms of DGK appears to have unique functions, and in general one isoform cannot replace another. It is therefore surprising that both DGKζ and DGKε appear to have the same protective effects and both apparently act by inhibiting the $G_{\alpha q}$ pathway. Further studies are required to completely understand this phenomenon.

4.5. Glucose Homeostasis

In rodents, glucose-induced insulin resistance is associated with increased intracellular DAG.[165] Insulin resistance is associated with obesity, and since DAG not only acts as a second messenger in signaling but also has a role in lipid metabolism as a precursor of triglycerides and phospholipids, it was not clear whether the DAG that accumulated during insulin resistance was involved in lipid signaling or in lipid metabolism. However, recent data indicate that the excessive DAG that accumulates in the insulin-resistant state has a direct signaling role in promoting insulin resistance. Moreover, it appears that DGKδ is involved in modulating the levels of this DAG. Chibalin et al. found abnormally low expression of DGKδ and reduced DGK

activity in skeletal muscle from type 2 diabetic patients.[166] In diabetic rodents, they found that they could rescue the reduced levels of DGKδ protein and DGK activity by normalizing their blood sugar. Together, these data suggested that DGKδ might be involved in glucose homeostasis. Indeed, in DGKδ haploinsufficient mice, which expressed about half of the normal levels of DGKδ, they found increased levels of DAG, glucose intolerance, and obesity. The underlying mechanism for DAG-induced insulin resistance in the DGKδ haploinsufficient mice is incompletely understood, but resistance appeared to be caused, in part, by increased PKC-mediated serine phosphorylation of insulin receptor substrate 1 and subsequent down regulation of glucose transport. It is not clear yet whether other DGKs participate in insulin signaling, but several of them, including DGKs α, ε, and ζ are expressed in skeletal muscle,[166] suggesting that they might act in this pathway as well.

4.6. Vision

Lipid intermediates of the PI cycle including DAG, PA, and PIP_2 are important secondary messengers in the vertebrate rod outer segment (ROS),[167] the membrane containing the photoreceptor, rhodopsin. This membrane has an unusual acyl chain composition with about half of the phospholipid acyl chains being docosahexanoic acid (22:6). These acyl chains are highly enriched in the principle phospholipids of this membrane, PC, PE, and PS. However, the lipids of the ROS that are intermediates in the PI cycle are present as minor components of just a few percent but have a very different acyl chain composition with little 22:6 and the predominant species being 18:0 and 20:4[168] as is found in other membranes.

Unlike verterbrates, transduction of the light signal in *Drosophila melanogaster* is initiated by phospholipase C-catalyzed hydrolysis of PIP_2 initiating the PI cycle. The lipid intermediates of this cycle including PIP_2, DAG, and PA have important roles in the phototransduction process for *Drosophila*.[169−171] DAG appears to have a dual role in the *Drosophila* retina, causing either stimulation of phototransduction through a PKC-independent pathway or a PKC-dependent inhibition of this process.[172] The importance of DGK in the retina of *Drosophila* is demonstrated by the finding that mutation of the *rdgA* gene, which encodes for *Drosophila* DGK2, an ortholog of mammalian DGKι, causes severe, light-dependent retinal degeneration. The mutation results in constitutive TRP channel activity[173] and appears to be involved with the DAG intermediate in the PI cycle.[174] The finding that there is no increase in the total DAG concentration in the *rdgA* mutants[175] may be a consequence of only a minor fraction of the total DAG changing concentration, that is, the DAG intermediate in the PI cycle. Another indication of the importance of DGK in retinal damage in *rdgA* mutants is the finding that loss of function mutations in a phosphatidic acid phosphohydrolase (Lazaro) reverses the *rdgA* mutant phenotype.[176,177] These results are consistent with PA being an important lipid signal for retinal degeneration. This is also suggested by the retinal degeneration observed in phospholipase D-null flies.[178] Since DGK-catalyzed phosphorylation of DAG is another pathway for PA production, DGKs are important for the functioning of the *Drosophila* retina.

Signal transduction in the vertebrate retina also involves an important role for DGK through down-regulating DAG as an activator of PKC. PKC regulates the activity of several proteins involved in the photocycle including the α and β subunits of transducin,[179] the γ-subunits of cyclic GMP-phosphodiesterase,[180] cyclic GMP-gated channel,[181] arrestin,[182] and rhodopsin.[183] Three different isoforms of DGK are highly expressed in mammalian retina in different studies. These isoforms are DGKε,[184,185] DGKγ,[25,186,187] and DGKι.[34] The presence of the various isoforms of DGK in the retina is interesting with regard to the acyl chain composition of lipid intermediates of the PI cycle. Because of its specificity for substrates with arachidonoyl groups, DGKε could contribute to the enrichment of lipid intermediates of the PI cycle with this acyl chain. This is particularly important in the retina where there is an abundance of docosahexanoyl acyl chains in the major phospholipid components but the exclusion of this acyl chain as a major constituent of PI cycle lipid intermediates.[168] Other isoforms that are expressed in the retina do not show acyl chain specificity. For example, the activity of DGKγ against dioleoyl glycerol is comparable to its activity against SAG and only slightly higher than its activity against didecanoyl glycerol.[25] Clearly DGK plays an important role in retinal function by facilitating certain lipid metabolic pathways. Because of the unusual acyl chain composition of the phospholipids of the retinal membrane, the acyl chain specificity of DGK isoforms is of particular importance. How this impinges on the roles of different DGK isoforms in this organ is yet to be determined.

5. INTER-RELATIONSHIPS

5.1. Role of PA Derived from DGK Activity

PA, itself, has a broad array of signaling properties that are very distinct from those of DAG. For example, PA can bind and regulate numerous proteins including phosphatidylinositol-4-phosphate 5-kinase,[188] RasGAP,[189] Raf-1 kinase,[190] p21-activated kinase 1,[191] mammalian target of rapamycin (mTOR),[192] atypical PKCs,[193] p47phox,[194] sphingosine kinase,[195] the transcriptional repressor Opi1p,[196] and the catalytic subunit of protein phosphatase-1.[197] As such, their ability to generate PA suggests that DGKs might also influence biological events not only by metabolizing DAG but also by producing PA. This would not be surprising based on what is known about PA signaling in plants. Seven DGK genes (AtDGK1−7) have been identified in *Arabidopsis thaliana*,[198] and in rice, there are eight putative DGK isoforms.[199] In plants, numerous PA targets have been identified,[200] and they vastly outnumber DAG targets, so it has been hypothesized that the primary role of DGKs in plants is to generate PA rather than to consume DAG.[201] PA in plants is usually produced in response to stress, suggesting that DGKs might influence the stress response. Supporting this possibility, expression of plant DGKs is induced in response to stresses such as wounding, chemicals, and fungal infection,[198,199] and overexpression of a rice DGK in tobacco plants enhanced the resistance of those plants to disease.[199] Although it is not clear exactly how plant DGKs are protective in conditions of stress, they are probably critical effectors in the stress response.

Given their potential role in PA signaling in plants, it was not surprising that mammalian DGKs appear to modulate proteins by producing PA. One example of this mechanism is the ability of DGKζ to modulate the activity of phosphatidylinositol-4-phosphate 5-kinase (PI4P5K) type Iα. The PI4P5K enzymes are potently activated by PA,[202] and DGK activity was found to co-immunoprecipitate with a complex that included a PI4P5K.[43] Together, these observations suggested that DGKs might modulate PI4P5K activity by generating PA. Indeed,

DGKζ colocalized and co-immunoprecipitated with PI4P5K type Iα, and its expression dramatically promoted the generation of PIP_2 in cells.[3] A kinase-dead DGKζ also co-immunoprecipitated with the PI4P5K but failed to enhance its activity. Collectively, these data indicate that localized PA generation, rather than a conformational change mediated by association of the PI4P5K with DGKζ, augmented PI4P5K activity.

In a separate study, DGKζ was shown to mediate DAG signaling downstream of the M1 muscarinic receptor (M1R), a seven-transmembrane receptor (GPCR).[11,41] As noted above, its translocation to M1R required binding to β-arrestins, which are scaffolding proteins that bind GPCRs. It was subsequently shown that PI4P5K type Iα also translocated to GPCRs by binding to β-arrestins, and its function at the GPCR was to promote internalization of the receptor.[42] Since DGKζ also binds β-arrestins, this collection of observations raises the possibility that DGKζ might function in this complex not only to metabolize DAG but also to promote PI4P5K activity by generating PA. This would provide a two-step mechanism to shut down the M1R receptor, where DGKζ first metabolizes DAG to reduce this signaling lipid and then the PA that it produces activates the PI4P5K enzyme in order to promote receptor internalization. This hypothetical model has not been specifically tested, but it agrees with data showing that transgenic overexpression of DGKζ in mouse myocardium protects the mice against cardiac hypertrophy initiated by excessive activation of a GPCR.[160]

DGK activity was also shown to co-immunoprecipitate with Rac1,[43] a member of the Rho family of GTPases that helps regulate changes in actin organization. It was subsequently demonstrated that DGKζ directly interacted with Rac1 and colocalized with it at sites of actin remodeling.[102] Overexpressing DGKζ induced neurite outgrowth that was inhibited by a dominant-negative Rac1, indicating that DGKζ promotes the activity of Rac1.[12] In an elegant series of experiments, Abramovici et al. recently demonstrated that DGKζ promotes Rac1 activity by producing PA, which activates p21-activated kinase 1 (PAK1).[47] PAK1 in turn, phosphorylates Rho guanine dissociation inhibitor (RhoGDI),[203] a protein that was also identified in DGK precipitates.[43] This phosphorylation of RhoGDI causes it to release Rac1, which enables its activation.[204] Supporting this mechanism, PAK1 phosphorylation was reduced and dissociation of RhoGDI from Rac1 was attenuated in DGKζ-deficient fibroblasts. These defects were rescued by exogenous PA and by expression of wild-type DGKζ but not by kinase-dead DGKζ. Additionally, DGKζ stably associated with both PAK1 and RhoGDI. Together, these data indicate that through its ability to produce PA, DGKζ has a major role in regulating Rac1-mediated signaling. A similar mechanism was recently demonstrated where, following activation of the c-Met receptor tyrosine kinase, DGKα modulates Rac1 activation and consequent membrane ruffling through its ability to produce PA. But unlike the example of DGKζ, the PA produced by DGKα activates atypical PKC isoforms ι and ζ, which presumably phosphorylate RhoGDI to relieve its inhibition of Rac1.[71]

The serine/threonine kinase mammalian target of rapamycin (mTOR) is an important intermediate in several pathways that manage cellular responses to environmental stress. Its activity is regulated, in part, by PA, which appears to compete with rapamycin for a binding site on mTOR. There is strong evidence indicating the phospholipase D (PLD) isoforms are largely responsible for providing the pool of PA that activates mTOR.[205] But there is evidence that DGKζ might also activate mTOR

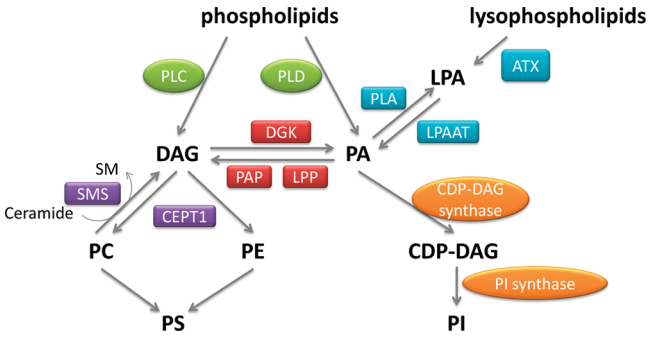

Figure 6. The main pathways implicated in DAG and PA synthesis. ATX, autotoxin; CEPT1, choline/ethanolamine phosphotransferase 1; LPAAT, LPA acyltransferase; LPP, lipid phosphate phosphatise; PAP, PA phosphohydrolase; SM, sphingomyelin; SMS, sphingomyelin synthase.

under some circumstances. For example, overexpression of DGKζ, but not DGKα, led to enhanced, serum-induced phosphorylation of p70 S6 kinase (p70S6K), a major downstream target of mTOR, and rendered the cells resistant to the effects of rapamycin.[75] Conversely, RNAi-mediated knockdown of DGKζ reduced phosphorylation of p70S6K. PA appeared to be important in this mechanism to activate mTOR, because DGKζ could not promote activation of a mutant mTOR that had reduced ability to bind PA. Collectively, these data indicate that DGKζ can activate mTOR, presumably through its ability to generate PA. The target of this PA, however, is not clear because another report showed in the same cell line that inhibiting PLD almost completely abolished serum-induced S6 kinase activity, indicating that PLD is largely responsible for activating mTOR.[192] It is possible then that instead of directly activating mTOR, DGKζ instead activates PI4P5Ks, which could provide PIP_2, an important activator of PLD enzymes.[206] Regardless of the mechanism, these data suggest that DGKζ can potentially activate mTOR and that it does so by producing PA.

Finally, there is evidence that DGKs might regulate additional cell responses through their ability to modulate the levels of PA. But the targets of PA in these cases are not as well-defined. For example, compound mutant mice lacking both DGKζ and DGKα have defects in T cell development that can be partially rescued by exogenous PA.[67] And defective Toll-like receptor (TLR) signaling in macrophages from DGKζ-deficient mice was rescued by addition of exogenous PA.[69] The role of PA is not clear, but it might be necessary to inhibit PI 3-kinases, which were excessively active in the DGKζ-deficient cells. Finally, a recent report suggested that PA derived from DGKα influenced neutrophil responses to antineutrophil cytoplasmic antibodies.[207] Collectively, these observations indicate that DGKs α and ζ regulate immune cell function not only by influencing DAG levels but also by producing PA.

5.2. Deacylation of PA To Form LPA Signals

Phospholipids can be modified by hydrolysis of one of the two acyl chains. In particular, phosphatidic acid, the product of DGK-catalyzed reactions, can be converted into lysophosphatidic acid (LPA) (Figure 6). In addition to the formation of LPA from PA by a phospholipase, LPA can also be formed by the action of autotaxin, an extracellular enzyme with phospholipase D activity that can convert other lysophospholipids to LPA.

dx.doi.org/10.1021/cr1004106 |*Chem. Rev.* 2011, 111, 6186–6208

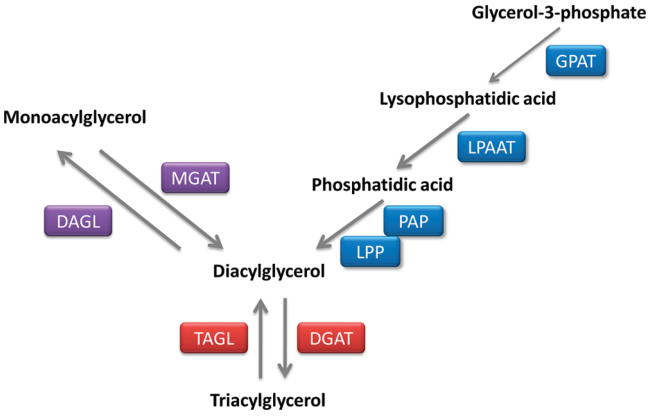

Figure 7. Acylation and deacylation of DAG. DAGL, DAG lipase; DGAT, diacylglycerol acyltransferase; GPAT, glycerol-phosphate acyltransferase; MGAT, monoacylglycerol acyltransferase; TAGL, TAG lipase.

LPA is involved in many cellular processes including cell proliferation, prevention of apoptosis, cell migration, cytokine and chemokine secretion, platelet aggregation, smooth muscle contraction, and neurite retraction. LPA has a particularly important role in brain where this lipid messenger has been found in significant concentration[208] and receptors for LPA are also found in both developing and mature brain.[209] The absence of LPA signaling results in defective brain cortical development.[210] The effects of LPA on cortical neurogenesis are through receptor-mediated intracellular calcium signaling.[211,212] LPA also binds to villin and inhibits actin regulatory functions of villin.[213] By this mechanism, LPA can also affect the cell cytoskeleton.

LPA signaling can result in several diverse pathologies. LPA accumulates in the central atheroma of human atherosclerotic plaques and is the primary platelet-activating lipid constituent of plaques.[214] LPA is also involved in prostate[215] and ovarian[216] cancer development. Lysophospholipids also have key regulatory roles in tumor angiogenesis.[217] The early phase of microglial activation is involved in *de novo* LPA production resulting in nerve-injury-induced neuropathic pain.[218,219]

There are two types of LPA, those with the acyl chain in the *sn*-1 position and those with the acyl chain in the *sn*-2 position. These are formed by phospholipase A_2 and by phospholipase A_1, respectively. In general these two types of LPA also differ by the 1-acyl-LPA having a saturated acyl chain and the 2-acyl-LPA being unsaturated.

LPAs play an important role in signal transduction in many diverse processes. They bind to seven different G-protein coupled receptors (GPCRs).[212,220] Although this complex group of signal transduction mechanisms has been elucidated, there is little information available with regard to the different affinities of structurally different LPAs for these receptors. One of the limitations of such studies is the relative chemical instability of 2-acyl-LPA because of intramolecular acyl group migration. However, the significant structural difference between 1-acyl-LPA and 2-acyl-LPA makes it almost certain that these two forms of LPA would have different affinities for specific receptor binding sites.

5.3. Acylation and Deacylation of DAG

Acylation and deacylation of DAG are the key reactions in the synthesis of triacylglycerols (TAG) and monoacylglycerols (MAG), which play important functions in a variety of processes in mammals such as intracellular energy storage, protection from the potentially toxic effects of excess of fatty acids, lactation, lipid transportation, and others.[221] A major pathway of TAG synthesis is the glycerol 3-phosphate (G-3-*P*) pathway, which begins with the acylation of G-3-*P* with a fatty acyl-CoA by enzyme glycerol-phosphate acyltransferase (GPAT), producing lysophosphatidic acid (LPA) (Figure 7). LPA can be further acylated by LPA acyltransferase (LPAAT) and dephosphorylated by phosphatidic acid phosphorylase (PAP) to yield diacylgycerol (DAG).[222] The final step is a reaction of converting DAG to TAG, catalyzed by diacylglycerol acyltransferase (DGAT). In this pathway, the acylation at the first position of the DAG chain depends on the subcellular location. Thus, in the mitochondria and peroxisomes only saturated fatty acids are added, whereas in the ER both saturated and unsaturated fatty acids are added.[223] The acylation at the second position of DAG by LPAAT takes place predominantly in the ER. Although different isoforms of LPAAT catalyze the same reaction, they have specificity for different acyl-CoA's.[224] Thus, it was shown that LPAATβ has the highest activity with 18:1, 18:2, and α18:3 acyl-CoA.[225]

The second pathway for TAG synthesis starts with the acylation of MAG with a fatty acyl-CoA by monoacylglycerol acyltransferase (MGAT), yielding DAG (Figure 7). This pathway is found in specific cell types, such as enterocytes, hepatocytes, and adipocytes,[226] and it plays an important role in storing energy in the form of TAG. So far, three isoforms of MGAT have been identified − MGAT1, MGAT2, and MGAT3. All isoforms are localized in the ER, although they differ in tissue expression pattern. Thus, MGAT1 was detected mainly in stomach, kidney, and adipose tissue, whereas MGAT2 and MGAT3 were found in the small intestine.[221]

These two pathways of TAG synthesis share the final step in converting DAG to TAG, catalyzed by diacylglycerol acyltransferase (DGAT).[222] There are two isoforms of DGAT enzymes in mammals, DGAT1 and DGAT2. DGAT1 is a member of the mammalian ACAT gene family,[227] and it is a part of membrane-bound O-acyltransferases (MBOAT).[228] DGAT2 is a member of a new family of acyltransferases that includes MGATs and wax monoester synthases. Both DGAT1 and DGAT2 also utilize MAG as a substrate in addition to DAG.[229] Several reports were published on the DGATs specificity for fatty acyl-CoA substrates of specific chain length and desaturation. Thus, it was shown that DGAT1 preferred a monounsaturated substrate, 18:1-CoA, as compared with saturated 16:0-CoA, whereas DGAT2 did not show such a preference.[230] In another study DGAT2 purified from *Mortierella ramanniana* showed preference for 12:0-CoA compared with 18:1-CoA.[231] When the activity was compared for DAGs, the enzyme exhibited higher activities with short- and medium-chain fatty acyl moieties, such as 6:0, 8:0, and 10:0, rather than longer chains, such as 12:0, 14:0, 16:0, and 18:0.[231]

Since TAG is the major energy store, it can be reconverted to DAG for further synthesis of complex lipids. The sequential deacylation of TAG is performed by specific lipases, and it results in the release of a fatty acid at each step with the generation of DAG, MAG, and glycerol. The first fatty acid is removed from TAG by adipose triglyceride lipase to produce DAG substrate, which is then hydrolyzed by DAG lipase to generate another fatty acid and MAG (Figure 7). As the final step, MAG is converted to

fatty acid and glycerol by MAG lipase.[232] Hydrolysis of DAG can be performed by DAG lipase (DAGL) at both *sn*-1 and *sn*-2 positions. DAGL was implicated in numerous signaling processes. Thus, in platelets upon thrombin stimulation, it produces arachidonic acid, facilitating further thromboxane and prostaglandin synthesis.[233] DAGLα and DAGLβ, two closely related *sn*-1 DAGL isoforms, are responsible for the synthesis of 2-arachidonoyl-glycerol (2-AG), an endocannabinoid that activates the cannabinoid receptor type 1. It was demonstrated that DAGLα and DAGLβ tissue distribution is different and that DAGLα is the major biosynthetic enzyme for 2-AG in the nervous system, whereas DAGLβ plays a more important role in regulating 2-AG levels in the liver.[234] It was also demonstrated that both DAGLα and -β −/− mice exhibit decreased levels of not only 2-AG but also free arachidonic acid, suggesting that DAGLs control levels of both lipids. Further, DAGLα was shown to play an essential role in regulating retrograde synaptic transmission and adult neurogenesis.[235]

Thus, pathways involving acylation and deacylation of DAG are a crucial part not only of TAG and MAG synthesis and, therefore, energy storage but also of many signaling cascades through endocannabinoid synthesis.

5.4. Other Sources of DAG: PA-Phosphohydrolases, PLC, Sphingomyelin Synthetase

Diacylglycerol (DAG) has unique functions in the cell, since it is not just a simple intermediate in lipid metabolism, but also an important membrane component and a key second messenger in multiple cellular signaling cascades. But there is a difference between the DAG that acts as a signaling lipid and the DAG that is an intermediate in lipid biosynthesis. It appears that the physiologically relevant DAGs are those containing a polyunsaturated acyl chain in the *sn*-2 position and that the DAGs composed of saturated fatty acids have weak signaling properties. Thus, it suggests that DAGs with different acyl chain compositions should come from different sources. Indeed, there are several enzymes catalyzing the same chemical reactions involving DAG, such as PA phosphohydrolase (PAP), lipid phosphate phosphatase (LPP), phospholipase C (PLC), and sphingomyelin synthase (SMS), proposing that DAG has distinct functions in the metabolic and signaling pathways.

PAP and LPP enzymes utilize two main *de novo* pathways of DAG synthesis in yeast and mammals.[223] Through the first pathway, DAG is synthesized from glycerol-3-phosphate, whereas in the second, it is synthesized from dihydroxyacetone-3-phosphate. Both pathways lead to the generation of LPA and then to PA, which is converted into DAG by PAPs and LPPs (Figure 6). LPPs are integral membrane proteins, previously known as Mg^{2+}-independent and *N*-ethylmaleimide-insensitive phosphatidate phosphatases (PAP2).[236] There are three LPPs (LPP1 and a splice variant LPP1a, LPP2, and LPP3), which hydrolyze a wide variety of lipids that include PA, LPA, sphingosine-1-phosphate, ceramide-1-phosphate, and diacylglycerol pyrophosphate.[237] The LPPs are localized in both plasma membrane and internal membranes. The active site of the LPPs is exposed on the outer surface of plasma membranes or the lumenal surface of internal membranes to facilitate the access of the lipid substrates to the LPPs, since lipid phosphates do not readily cross membranes.[238]

In contrast to LPPs, PAPs have specificity for phosphatidic acid only. Based on information about sequence homology, a group of mammalian enzymes called lipins were recognized to have PAP activity. Three mammalian lipins were found (lipin-1 with two alternative splicing variants, lipin-1A and lipin-1B, lipin-2, and lipin-3), all of which strictly require Mg^{2+} for activity.

In addition to *de novo* synthesis, there are three alternative pathways for DAG production: by sphingomyelin synthase, by phospholipase C, and through the phospholipase D pathway (Figure 6). Phospholipase D (PLD) metabolizes glycerophospholipids, such as PC, PI, and PE, to produce PA, which can be converted to DAG by PAP or LPP. DAG generated through the PLD−PAP pathway predominantly contains saturated fatty acids; therefore it is generally believed that the main PAP function is to serve in lipid biosynthesis or to terminate PA signaling when coupled with PLDs and not to initiate DAG signaling.

Sphingomyelin synthase (SMS) catalyzes the transfer of phosphocholine from PC onto ceramide, yielding sphingomyelin and DAG as a side product. Two SMS genes, SMS1 and SMS2, have been cloned.[239] SMS1 is located in the *trans*-Golgi apparatus, whereas SMS2 is mostly located at the plasma membrane. It was also shown that SMS1 and SMS2 expression positively correlate with levels of sphingomyelin in lipid rafts and that SMS1 has been implicated in the regulation of sphingomyelin level in the lipid rafts and raft functions such as endocytosis and apoptosis.[240]

Phospholipase C (PLC) catalyzes the production of DAG by hydrolyzing the phosphate bond on glycerophospholipids, such as PC and PI. Interestingly, mammalian PC-specific PLC has not yet been cloned and fully characterized, although a number of studies demonstrated the expression of PC-PLC isoforms in mammalian cells.[241] Another much better characterized PLC, PI-specific PLC, is a key enzyme in the DAG generated signaling processes. To date, 13 mammalian PLC isoforms have been identified and categorized into six groups: PLC-β, -γ, -δ, -ε, -ζ, and -η.[242] Induction of PLC activity not only increases membrane DAG levels but also forms inositol-1,4,5-triphosphate, which leads to the release of Ca^{2+} from the ER, thus activating several signaling cascades. DAG generation by PI-PLC is highly dependent on extracellular stimuli, and the DAG produced acts as a second messenger and is not usually consumed for metabolic purposes.[243]

5.5. PA vs DAG for Phospholipid Synthesis

There are two pathways for the synthesis of the major phospholipids of mammalian cells (Figure 6). One is by the activation of PA through its reaction with CTP to produce CDP-DAG that then is transformed by reaction with inositol to yield PI. The other pathway involves the activation of serine, choline, or ethanolamine with CTP to produce a CDP-alcohol that in turn reacts with DAG to yield PS, PC, or PE. Thus to produce PI, PA is activated, while to produce PC, PE, or PS, the headgroup alcohol is activated. Of course different enzymes are involved in these two pathways for phospholipid synthesis, and they are also distinguished by producing lipids with different acyl chains. In the case of PI, it is predominantly stearoyl or arachidonoyl lipids, while with PC, PE, and PS, there is a larger variety of acyl chains. Interestingly, the substrate of the DGK-catalyzed reaction, DAG, is also a substrate for the enzymes that synthesize PC, PE, and PS, while the product of the DGK-catalyzed reaction, PA, is the

substrate for making CDP-DAG, which then goes on to make PI. Hence, the interconversion of lipids catalyzed by DGK could also contribute to determining the relative proportion of different lipid classes in the membrane.

6. CONCLUSIONS AND FUTURE DEVELOPMENTS

DGKs play an important role in cell signaling. They catalyze the conversion of one lipid signal, DAG, to another, PA. In addition to the importance of DAG and PA to signaling, these lipids are also the precursor of other lipid signals including lysophosphatidic acid and 2-arachidonoyl glycerol, both of which bind to their own specific GPCRs. Not only is the reaction catalyzed by DGKs a focal point for several signaling pathways, but it is also a step in several lipid biosynthetic pathways, perhaps the most important of which is in the synthesis of phospholipids. In particular, the reaction catalyzed by DGK is the first step in synthesis of PI through the PI cycle, thus having importance both as a pathway for lipid synthesis and for signal transduction through phosphorylated forms of PI. The synthesis of DAG also can occur through the action of sphingomyelin synthetase, thus providing a site of interaction between glycerolipid synthesis and sphingolipid pathways. DAG is also a precursor for triglycerides, as well as stimulating the formation of lipid droplets. Hence, in the absence of activated DGK, more DAG will be converted to triglycerides and will form lipid droplets.

Different isoforms of DGK affect these metabolic or signaling pathways to different extents. These differences come about because of expression of specific isoforms in different organs, different subcellular localization, and interaction with different proteins that may be part of other signal transduction or metabolic pathways, different modes of activation of DGK isoforms as well as different intrinsic catalytic properties of these isoforms. With regard to the last factor, there is only one documented example of a different intrinsic catalytic property of a DGK isoform. It is the specificity of DGKε for arachidonoyl-containing substrates. This specificity also suggests a special role of DGKε in PI cycling since the lipid intermediates in this pathway are rich in the acyl chains that preferentially interact with DGKε.

DGKs are known to modulate many cellular functions including cell proliferation, as well as cell morphology. There is no established pathology that is caused by the lack of a functioning DGK isoform, but several pathological consequences have been observed as a result of knocking out a single isoform. Additionally, the resulting phenotype of the knockout animals is different for different DGK isoforms. Specific examples have been given in this review and include immune, brain, and cardiac function, as well as vision.

There is potential for developing therapies based on the modulation of DGK activity. There have been several suggestions for using DGK as a drug target in disease.[115,139,244,245] The importance of DGKε and lipid signaling in epilepsy has been shown, suggesting that modulation of the activity of DGKε could provide a novel drug target for this disorder.[136] Another brain disorder for which DGK has been implicated is bipolar disease. The gene for DGKη is most altered in bipolar disease resulting in decreased concentrations of DAG and lower activation of PKC.[246] DGKζ is the predominant DGK isoform in heart and its activity has been shown to affect cardiac function. This isoform has been suggested to be a target for diabetic cardiomyopathy,[162] atrial fibrillation,[164] left ventricular remodeling,[159] heart failure,[163] and cardiomyocyte hypertrophy.[247] DGKε has also been shown to have importance in restoring heart function under chronic pressure overload.[156] There is also evidence that DAG regulates the level of the guanine nucleotide exchange factor, Ras GRP, and that in CD8+ T cells the level of DAG is regulated by DGKζ. As a consequence, mice with DGKζ-deficient T cells exhibit enhanced digital signaling of RasGRP and grew smaller tumors but with no signs of any autoimmune disease. It was suggested that DGKζ is a novel target to enhance antitumor immunity. Additionally, because of the well-known involvement of DAG in cell proliferation, DGK inhibitors have been sought for use in cancer chemotherapy.[248] The type II DGK inhibitor, R59022, suppresses cell polarity in Walker carcinosarcoma cells and inhibits cell locomotion by increasing DAG concentrations.[249] With regard to cell motility, DGK is required for cell invasiveness and anchorage-independent growth in estrogen receptor negative breast cancer cells. DGK has been suggested as a target to limit the invasiveness of such tumors.[250] Some hypoglycemic agents activate DGK to lower DAG levels. It has been suggested that new therapeutic agents for diabetic nephropathy could target inhibition of the DAG−PKC extracellular signal-regulated kinase pathway.[251] This same group also showed that α-tocopherol enhanced DGK activity and could prevent glomerular dysfunction in diabetes.[252] It was suggested that DGK activation could be used in the treatment of diabetic nephropathy by inhibiting the DAG−PKC pathway. Administration of α-tocopherol was also shown to improve insulin sensitivity on skeletal muscle. Both α-tocopherol and ω-3 fatty acids activated DGK and inhibited phosphatidate phosphorylase resulting in the reduction of DAG and enhanced insulin sensitivity.[253]

It can thus be appreciated that there is a wide variety of situations for which modulation of DGK activity can be a potential drug target. This is because the lipid substrate and product, DAG and PA, are modulators of many signal transduction pathways. However, the final application of DGK inhibitors or activators to the clinic is currently nonexistent. There are a number of complexities in developing specific drugs for this purpose. There are 10 isoforms of mammalian DGK, each having some unique function, as well as some common overlapping functions. It is therefore difficult to have a specific target since it has been difficult to design inhibitors for individual isoforms and even if this is accomplished, there will be remaining isoforms with some overlap of function that can substitute for the therapeutic target. Nevertheless, there is still the possibility in the future to design drugs that will affect specific isoforms of DGK and target them to tissues in which these drugs will have a dominant effect.

There are no known diseases for which mutation of a DGK isoform is responsible. However, with 10 different isoforms and some overlap of function among them, such defects would make it difficult to identify the situations in which such a mutation would be more likely. Also in many cases defects in one DGK isoform may not lead to an identifiable pathology in basal conditions but nevertheless result in pathology under certain circumstances.

It is clear that DGK is a potential drug target. However, its importance in medicine has not been fully evaluated or

exploited because of some of the complexities alluded to above. Nevertheless, we believe that its importance as a drug target will be realized in the coming years.

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Richard M. Epand received his A.B. from the Johns Hopkins University and his Ph.D. in Biochemistry from Columbia University. He then gained research experience in the laboratories of Harold Scheraga at Cornell University and with Professor Luis Leloir at the Instituto de Investigaciones Bioquímicas in Buenos Aires. Dr. Epand is currently a Professor in the Department of Biochemistry and Biomedical Sciences at McMaster University in Hamilton, Ontario, Canada. Dr. Epand has received the Avanti Award in Lipids from the Biophysical Society and has been elected a Fellow of that society. He has also been granted a Senior Investigator Award from the Canadian Institutes of Health Research. Dr. Epand is currently the Editor-In-Chief of *Chemistry and Physics of Lipids*. He has over 450 manuscripts published in peer reviewed journals as well as having edited several books. The research interests of Dr. Epand's laboratory have focused on the properties of membranes and on the interaction between membrane proteins and the surrounding lipids with a goal of understanding how these interactions modulate biological function.

ABBREVIATIONS

Cbl	E3 ubiquitin ligase
DAG	diacylglycerol
DAGL	DAG lipase
DGAT	diacylglycerol acyltransferase
DGK	diacylglycerol kinase
EGFR	epidermal growth factor receptor
EGFR-Ub	ubiquitinated EGFR
ER	endoplasmic reticulum
GAP	GTPase-activating protein
GPCR	G-protein-coupled receptor
LPA	lysophosphatidic acid
LPAAT	LPA acyltransferase
LPP	lipid phosphate phosphatase
LTP	long-term potentiation
MAG	monoacylglycerol
MARCKS	myristoylated alanine-rich C kinase substrate
MEF	mouse embryonic fibroblasts
MGAT	monoacylglycerol acyltransferase
mGluR	G-protein-coupled glutamate metabolic receptor

PA	phosphatidic acid
PAP	PA phosphohydrolase
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PI	phosphatidylinositol
PI cycle	phosphatidylinositol cycle
PIP_2	phosphatidylinositol-4,5-bisphosphate
PI3K	phosphatidylinositol 3-kinase
PI4P5K	phosphatidylinositol-4-phosphate 5-kinase
PKC	protein kinase C
PLC	phospholipase C
PLD	phospholipase D
PS	phosphatidylserine
RasGRP	Ras-guanyl-nucleotide-releasing protein
SAM	sterile alpha motif
SAG	1-stearoyl-2-arachidonoyl glycerol
SAPA	1-stearoyl-2-arachidonoyl phosphatidic acid
SMS	sphingomyelin synthase
TAG	triacylglycerol
USP8	ubiquitin-specific protease 8

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