

REVIEW ARTICLE

Diacylglycerol kinases: at the hub of cell signalling

Isabel MÉRIDA*¹, Antonia ÁVILA-FLORES† and Ernesto MERINO*

*Department of Immunology and Oncology, Centro Nacional de Biotecnología/CSIC, Darwin, 3, UAM Campus Cantoblanco, E-28049 Madrid, Spain, and †Servicio de Inmunología, Hospital de la Princesa, Universidad Autónoma de Madrid, E-28049 Madrid, Spain

DGKs (diacylglycerol kinases) are members of a unique and conserved family of intracellular lipid kinases that phosphorylate DAG (diacylglycerol), catalysing its conversion into PA (phosphatidic acid). This reaction leads to attenuation of DAG levels in the cell membrane, regulating a host of intracellular signalling proteins that have evolved the ability to bind this lipid. The product of the DGK reaction, PA, is also linked to the regulation of diverse functions, including cell growth, membrane trafficking, differentiation and migration. In multicellular eukaryotes, DGKs provide a link between lipid metabolism and signalling. Genetic experiments in *Caenorhabditis elegans*, *Drosophila melanogaster* and mice have started to unveil the role of members of this protein family as modulators of receptor-

dependent responses in processes such as synaptic transmission and photoreceptor transduction, as well as acquired and innate immune responses. Recent discoveries provide new insights into the complex mechanisms controlling DGK activation and their participation in receptor-regulated processes. After more than 50 years of intense research, the DGK pathway emerges as a key player in the regulation of cell responses, offering new possibilities of therapeutic intervention in human pathologies, including cancer, heart disease, diabetes, brain afflictions and immune dysfunctions.

Key words: cancer, diabetes, diacylglycerol kinase, lipid kinase, phosphatidic acid, Ras signalling.

INTRODUCTION

The first description of an enzyme activity that catalysed synthesis of PA (phosphatidic acid) by phosphorylating DAG (diacylglycerol) goes back to 1959, in the pioneering studies by Hokin and Hokin [1]. In 1990, a review by Kanoh et al. [2] provided the first clues of distinct DGK (diacylglycerol kinase) species having essential functions in the regulation of cell responses. Today, almost 50 years after the initial observations, the DGK enzymes are a large family of lipid kinases that represent a lively rapidly progressing research area. In the present review, we will revisit the main discoveries over the years, from the cloning of the first DGK isoform to the lessons offered by genetically modified animal models and early clues relative to the role of these enzymes in human disease.

FIFTY YEARS OF DGK RESEARCH

The studies by Hokin and Hokin in the 1950s [3], describing acetylcholine-induced exchange of inositol and phosphate, identified what was initially termed the “phospholipid effect” and led to characterization of the PI (phosphatidylinositol) cycle. Early in their work, they recognized a kinase activity that catalysed the phosphorylation of DAG into PA as an intrinsic component of this cycle. Whereas the initial studies in erythrocytes described

DGK as an activity present in membranes [4], subsequent work identified DGK activity in the cytosol of rat brain [5]. From then on, studies by several groups described agonist-dependent regulation of DGK, suggesting the existence of differentially regulated activities [6–8]. It was not until 1983 that Kanoh et al. [9] first reported the purification from pig brain of an 80 kDa protein with DGK activity. An antibody generated against this purified DGK demonstrated tissue specificity [10], translocation to the membrane and regulation by phosphorylation [11], three characteristics that are present in most members of the DGK enzyme family. At about the same time, the absence of DGK activity from photoreceptor cells of *Drosophila* vision mutants was reported, showing a functional effect due to DGK loss [12], and Bell's group reported the characterization of DGK from *Escherichia coli* [13] (Figure 1).

The identification of two DGK inhibitors [14] provided new clues to the function of this activity in regulation of cell responses. Inhibitor treatment increased platelet secretion and aggregation responses to submaximal concentrations of thrombin [15]. These effects correlated closely with a rise in DAG and decreased PA production, with no changes in Ca²⁺ mobilization. Additional studies demonstrated the participation of DGK in multiple events, including spontaneous hypertension in rats [16], endothelial cell mitogenesis [17], superoxide production in neutrophils [18] and IgE-induced histamine release by mast

Abbreviations used: AICD, activation-induced cell death; ALCL, anaplastic large cell lymphoma; AP-1, activator protein 1; C1 domain, type 1 conserved domain; CERK, ceramide kinase; DAG, diacylglycerol; DGK, diacylglycerol kinase; EGF, epidermal growth factor; ERK, extracellular-signal-regulated kinase; FcεRI, high-affinity receptor for IgE; GEF, guanine-nucleotide-exchange factor; GFP, green fluorescent protein; GPCR, G-protein-coupled receptor; HGF, hepatocyte growth factor; HIF, hypoxia-inducible factor; IL, interleukin; JAK, Janus kinase; LAP, localized aggressive periodontitis; LV, left ventricular; MAPK, mitogen-activated protein kinase; MARCKS, myristoylated alanine-rich C-kinase substrate; mGluR, metabotropic glutamate receptor; MI, myocardial infarction; M₁R, muscarinic type 1 receptor; mTOR, mammalian target of rapamycin; NF-κB, nuclear factor κB; NLS, nuclear localization sequence; PA, phosphatidic acid; PFK, phosphofructokinase; PI3K, phosphoinositide 3-kinase; PIPkin, phosphatidylinositol phosphate kinase; PKC, protein kinase C; PLC, phospholipase C; PLD, phospholipase D; PPARγ, peroxisome-proliferator-activated receptor γ; pRb, retinoblastoma protein; PS, phosphatidylserine; RasGRP, Ras guanyl-nucleotide-releasing protein; siRNA, small interfering RNA; SPK, sphingosine kinase; STAT, signal transducer and activator of transcription; TCR, T-cell receptor; TLR, Toll-like receptor; TNFα, tumour necrosis factor α; TRP, transient receptor potential; TRPC, canonical TRP channel; UNC13, uncoordinated 13; Munc13, mammalian UNC13 homologue; VEGF, vascular endothelial growth factor.

¹ To whom correspondence should be addressed (email imerida@cnb.uam.es).

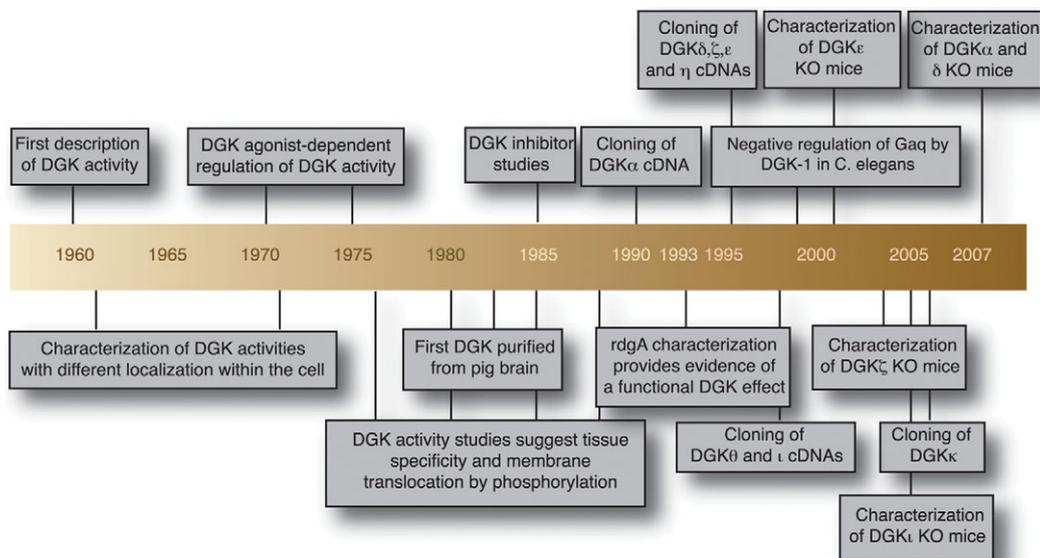


Figure 1 Timeline

Main contributions to the DGK field in the last 50 years. KO, knockout.

cells [19]. At that time, the major role assigned to DGK was the control of intracellular DAG levels, and the biological effects of DGK inhibitors were interpreted as a result of PKC (protein kinase C) overstimulation. These early studies suggested similar DGK and PKC properties with respect to DAG utilization, PS (phosphatidylserine)-dependency and protein translocation from soluble fractions to membranes, an event reported for DGK in rat brain [20], human neutrophils [21] and amoebae [22].

In 1990, the sequence of the soluble 80 kDa porcine DGK was reported [23], together with the cloning of the human orthologue [24]. Sequence analysis identified a putative ATP-binding site, two cysteine-rich zinc finger-like sequences similar to the C1 (type I conserved) domain in PKC, and two EF-hand motifs characteristic of Ca²⁺-binding proteins. Accordingly, the 80 kDa DGK was reported to bind Ca²⁺ and phospholipids and to undergo Ca²⁺-regulated translocation to membranes [25]. Tissue expression analysis revealed high expression of the 80 kDa DGK isoform in oligodendrocytes, thymus and peripheral T-cells, suggesting specific roles for this enzyme in these tissues. The cloning of this first DGK isoform (later identified as DGK α) facilitated characterization of two additional isoforms, originally named DGKII (β , predominantly located in neurons) [26] and DGKIII (γ , also abundantly expressed in brain) [27]. The cloning of the protein encoded by the *rdgA* gene in *Drosophila* demonstrated the existence of additional DGK isoforms with different regulatory domains [28]. Throughout the 1990s, six additional mammalian isoforms were cloned, and a tenth isoform has since been reported [29].

DGK evolution as regulators of lipid metabolism and signalling

Mammalian DGKs comprise an extended family, currently with ten members classified into five different subtypes on the basis of the presence of different regulatory domains in their primary sequences (Figure 2). DGK diversity is increased further by alternative splicing, which produces several isoforms with distinct domain structures [27,30–34]. The three mammalian type I DGK have characteristic Ca²⁺-binding EF-hands and a recoverin-like motif in the N-terminus, whereas the two type II isoforms have a

PH (pleckstrin homology) domain. Members of the type IV group contain C-terminal ankyrin repeats and a PDZ-domain-binding sequence, as well as MARCKS (myristoylated alanine-rich C-kinase substrate) homology region upstream of the catalytic site. The single type V member has a Rho-binding domain, and the only type III member has the simplest structure, with no regulatory region. Proteins of this family are conserved in multicellular organisms, including *Dictyostelium discoideum*, which has a single gene (*dgkA*) that encodes an enzyme related to mammalian DGK θ [35,36]. Disruption of the *dgkA* locus alters myosin II assembly, raising the intriguing possibility that DAG and/or PA may have a role in controlling cytoskeletal organization in this organism. Analysis of *Drosophila melanogaster* or *Caenorhabditis elegans* genomes reveals the presence of members for each of the different DGK subtypes, suggesting non-redundant functions. These two biological models have contributed greatly to build knowledge in the DGK field, making important inroads for understanding the role of mammalian enzymes.

DGK activity has also been reported in several plant species, including tobacco, wheat, tomato and *Arabidopsis*. Seven candidate genes that encode putative DGK isoforms are present in the *Arabidopsis* genome. Plant DGKs fall into three distinct clusters, simpler in organization than mammalian DGKs since none contains a regulatory region. Cluster I DGK contains two cysteine-rich domains, whereas clusters II and III have only the characteristic catalytic region [37]. The major role of DGK in plants is related to PA generation in response to biotic challenges, such as microbial elicitation, and abiotic stress, including chilling, salts, drought and dehydration [38–40]. DGK-derived PA levels also accumulate during various developmental processes, including root elongation [41].

Phosphorylation of DAG into PA also occurs in bacteria, but prokaryotic DGKs are homotrimeric helical integral membrane proteins [42], different from the large family of cytosolic proteins present in multicellular organisms. Prokaryotic DGKs play an important metabolic role by converting the DAG produced under conditions of environmental stress into PA [43,44]. Thus, whereas the same enzymatic activity is present from bacteria to mammals, in multicellular organisms, this activity is not exerted

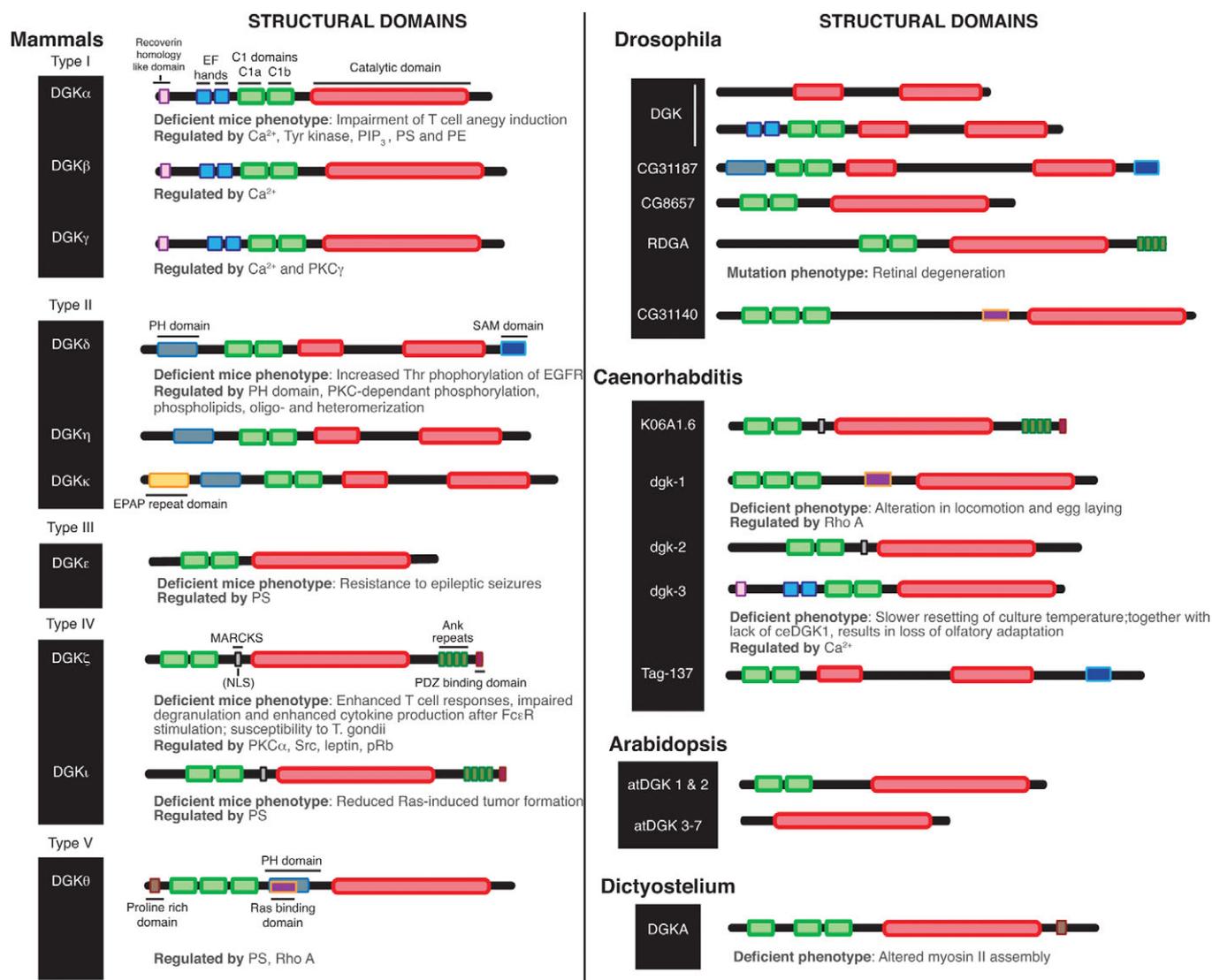


Figure 2 The DGK family

The different DGK isoforms present in multicellular organisms are represented, indicating the distinct regulatory domains. Regulation mechanisms and the phenotype caused by DGK deficiency/mutations are indicated. Ank, ankyrin; atDGK, *Arabidopsis thaliana* DGK; ceDGK1, *C. elegans* DGK1; EGFR, EGF receptor; PE, phosphatidylethanolamine; PIP₃, PtdInsP₃; SAM, sterile α -motif.

by membrane enzymes, but by proteins that can only reach the membrane in response to external stimuli. The acquisition of this capacity permits DGK to participate in receptor-initiated signalling pathways, positioning these enzymes to act as a perfect link between signalling and metabolism.

Conserved DGK domains: the DGK 'signature'

Analysis of the mammalian DGK primary sequence reveals conserved domains that provide the DGK family signature. The C-terminal half of the protein houses the catalytic domain, which is regulated by anionic phospholipids such as PS [45–49]. This domain is subdivided into a conserved motif called DAGKc in SMART (<http://smart.embl-heidelberg.de/>), which contains the sequence $\Phi\Phi\Phi\text{CGGDGT}$ (Φ represents any hydrophobic residue), and an accessory domain (SMART ID DAGKa). The identification of an inactivating mutation in the *Drosophila* rdgA sequence pointed to the GGDG motif as the putative ATP-binding

region. The DAGKc domain is present in two other families of lipid kinases, SPK (sphingosine kinase) and CERK (ceramide kinase) [50,51]. The SPK catalytic site contains a conserved SGDG motif reminiscent of the GGDG DGK sequence. Site-directed mutagenesis and covalent modification with an ATP analogue demonstrated that this motif represents the nucleotide-binding region of SPK [52]. Mutation of the second guanine residue in the GGDG motif of DGK abolishes its kinase activity further, confirming the essential role of this motif [53–55]. Although DGK shares the DAGKc region with SPK and CERK, it is highly specific for DAG as substrate and does not catalyse phosphorylation of sphingosine or ceramide, suggesting that the DAGKa domain is responsible for substrate recognition. The catalytic regions of these three lipid kinase families show some sequence conservation with the catalytic domains of PFK (phosphofructokinase) and PPNK (polyphosphate/NAD⁺-ATP kinase) [56]. These studies suggest that these kinases, albeit distantly related, may all share a similar ATP-binding site and

catalyse phosphorylation of their substrates using a similar mechanism. Recently, an extensive review has summarized the properties of these structurally related lipid kinases [57].

Site-directed mutagenesis of conserved residues in human DGK α has helped to identify additional amino acids that are critical for catalysis. Six aspartate residues along the C-terminal domain are conserved in all DGKs, five in sequences that have homology with the PFK catalytic site [58]. Mutation of any of these residues results in reduced or no catalytic activity, indicating that the catalytic domain comprises the entire C-terminal region. Some of these mutations alter PS-dependent activation, suggesting some type of direct PS interaction with the catalytic region.

All DGK family members contain at least two C1-type motifs homologous with the PKC phorbol ester/DAG-binding regions [59]. The presence of C1 domains in the DGK sequence originally led to consideration of these motifs as being responsible for DAG binding. Nevertheless, sequence analysis indicated that, with the exception of the first C1 domain in DGK β and DGK γ [60], the C1 regions lack the key residues that define a canonical C1-like phorbol ester-binding domain [61]. The participation of DGK C1 domains in the enzymatic activity of this family remains a matter of debate. Some studies have suggested that these conserved motifs are required for activity [62], whereas others have found them dispensable for DAG phosphorylation *in vitro* [46]. Some well-characterized plant DGKs lack C1 domains, suggesting that these domains are not necessary for activity [63]. Whereas the DGK C1 domains may not be needed for catalytic DAG binding, they do appear to be critical for membrane targeting. Mutations that disrupt one of the C1 domains impair receptor-dependent translocation to the plasma membrane of GFP (green fluorescent protein)–DGK ζ chimeras in response to G-protein-coupled receptors [64]; this is also the case for DGK θ [65] and for DGK γ [66]. Targeting to the membrane may be fostered by C1 domain interaction with lipids and/or proteins. Accordingly, DGK C1 domains are proposed to bind PI3K (phosphoinositide 3-kinase) derivatives, β -arrestin and Rho [67–69].

To date, there is no information on the three-dimensional structure of DGKs. Such studies would greatly help to determine the exact region where the DAG binds, the domains that contact the membrane and the mechanism governing the transfer of substrates from the lipid bilayer to the catalytic pocket.

DGK regulatory domains dictate the ‘when’ and ‘how’ of enzyme action

Whereas all DGK catalyse the same reaction, the presence of non-conserved regulatory regions appears to dictate subtype-specific DGK activation. These regions confer specificity to the distinct DGK isoforms by restricting their subcellular site of action and/or defining their activation mechanism (Figure 2). The presence of specific localization signals, such as NLSs (nuclear localization signals) in DGK ζ or DGK ι permit their activity in the nucleus [54]. The interaction with certain partners may add further constrictions to DGK function, such as the recently described interaction of DGK ζ with SNX27 (sorting nexin 27) that allows DGK ζ to mediate endosomal recycling [70].

Most DGKs are cytosolic in unstimulated cells and translocation to membranes appears to be a general mechanism that modulates the spatiotemporal activation of this family. Then non-conserved specific domains link DGK translocation/activation to signals elicited by receptor triggering. The presence of EF-hands and recoverin regions confer Ca²⁺-sensitivity to type I DGKs, coupling their activation to receptors that elicit PLC (phospholipase C)-mediated Ca²⁺ elevation. The MARCKS domain in DGK ζ also provides PLC-dependent membrane-tar-

geting mechanism, but, in this case, it is mediated through PKC-dependent phosphorylation [64,71,72]

Translocation from the cytosol to the nucleus has been reported for different DGKs, including DGK α , DGK γ , DGK θ , DGK ζ and DGK ι [73–77]. Except for the type IV DGKs, none of the other isoforms contains a canonical NLS, suggesting the existence of alternative mechanisms controlling nuclear transportation. In this regard, it has been proposed that, at least in DGK γ , the entire C1 domain acts as an NLS [77]. Nuclear localization of DGKs is probably related to the control of DAG in this particular compartment [78]. Some recent reviews have summarized the latest results regarding DGK function in the nucleus [79,80].

The presence of specific motifs provides an exquisite DGK regulation that is translated into controlled changes in the levels of membrane bioactive lipids regulated by this activity. The subtle modulation of DAG and PA levels is exclusively sensed by a restricted set of molecules, providing the means for the high level of specificity of DGK-regulated responses.

DGK function: from DAG attenuators to PA producers

From early on, the main function attributed to the DGK family has been that of negative regulation of DAG receptors. DGKs were initially recognized as modulators of classical and novel PKC family members. Some studies have pointed out that some DGKs form a complex with certain DAG-sensitive PKC isoforms, leading to a mutual regulation of both enzymes. Some examples would be DGK γ association with PKC γ [81], DGK θ with PKC ϵ [65] and DGK ζ with PKC α [82].

DGK function has expanded with the characterization of additional families of DAG-regulated proteins. Among others, recent studies have shown negative regulation of UNC13 (uncoordinated 13) by DGK-1 in *C. elegans* (DGK θ orthologue) [83,84], of RasGRP (Ras guanyl-nucleotide-releasing protein) 1 by DGK α and DGK ζ [85,86], of RasGRP3 by DGK ι [87] and of β_2 -chimaerin by DGK γ [88] (Figure 3).

Whereas no PA-binding domain has yet been formally characterized, PA-mediated binding is proposed to cooperate in membrane localization and activation of several proteins, including phosphodiesterases [89], Raf-1 [90], some phosphatases [91] and, more recently, the Ras GEF (guanine-nucleotide-exchange factor) Sos (Son-of-sevenless) [92]. PA binds and activates the mTOR (mammalian target of rapamycin), a master regulator of cell growth [93]. The interaction between PA and mTOR is abolished by rapamycin, suggesting that rapamycin action results from preventing PA-dependent mTOR modulation [94,95]. In plants, various PA targets have been identified, including PDK1 (phosphoinositide-dependent protein kinase 1), PEPC (phosphoenolpyruvate carboxylase), Hsp90 (heat-shock protein 90) and 14-3-3 proteins, among others [96,97]. In yeast, PA binding maintains the transcriptional regulator of phospholipid synthesis, Opi1p, at the endoplasmic reticulum, preventing its nuclear localization [98] (Figure 3).

Generation of PA through DAG phosphorylation also represents the first step in PI resynthesis; it is thus widely accepted that DGKs are crucial components of PI turnover (see Figure 7). Studies by various groups showed that PA generation controls the PI cycle via direct recycling into PtdIns(4,5)P₂ or through activation of molecules involved in PI turnover, such as mammalian type I PI4P5K (phosphatidylinositol 4-phosphate 5-kinase) [type I PIPkin (phosphatidylinositol phosphate kinase)] [99].

Independently of their lipid kinase activity, it is likely that DGK could have scaffold functions. For instance, a previous study suggests that DGK ζ acts as a Rac regulator in neurons, forming a complex with syntrophin and RacGDP in the cytosol.

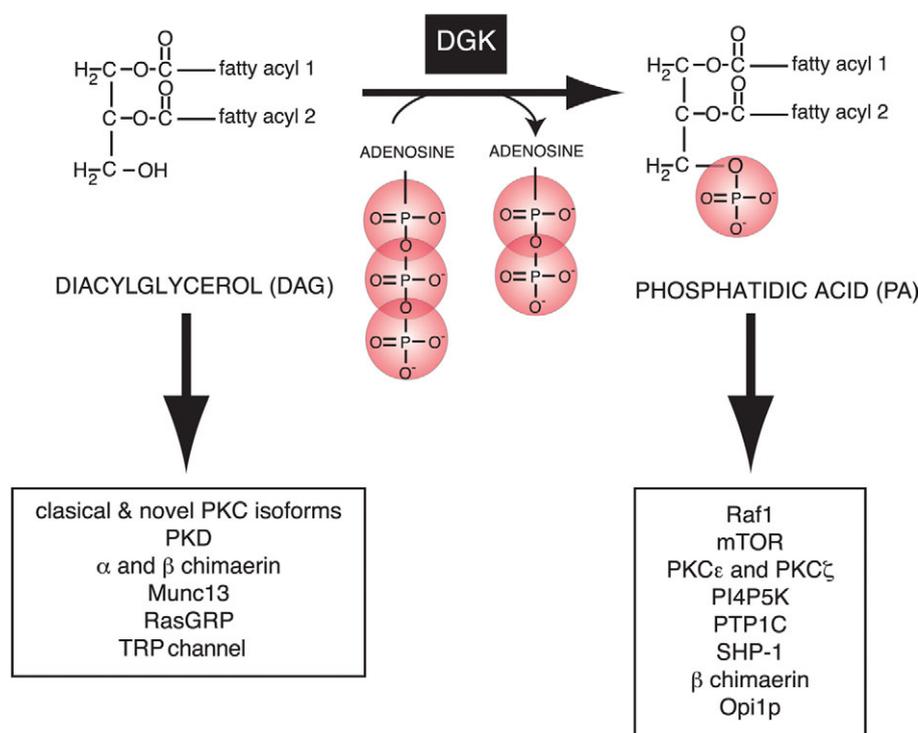


Figure 3 DGK enzymatic activity regulates levels of DAG and PA at the membrane

The receptors for each bioactive lipid are summarized. PTP1C, protein tyrosine phosphatase 1C; SHP-1, Src homology 2 domain-containing protein tyrosine phosphatase 1.

PKC-mediated phosphorylation of the DGK ζ MARCKS domain would favour translocation of DGK ζ , together with the rest of the complex, to the membrane, where nucleotide exchange on Rac would take place [100].

Tissue specificity of DGK expression

DGKs are expressed ubiquitously, although some isoforms show a distinct tissue distribution, being most abundantly expressed in brain and haemopoietic tissues [101]. It is not surprising to find such specificity for these tissues, in which non-kinase DAG-regulated proteins are also expressed abundantly. The RasGRP family of GEFs for Ras, the chimaerin family of GAP for Rac, and the Munc13 family (mammalian homologue of UNC13) are all expressed predominantly in brain and haemopoietic tissue, suggesting that DAG-based regulatory mechanisms are particularly relevant in these systems. This correlation between expression of DGK and DAG-regulated proteins in mammals is not observed in plants. In *Arabidopsis*, for instance, the presence of multiple DGK isoforms contrasts with its single C1 domain-containing protein. This suggests that, at least in plants, the main function of DGK is not to attenuate DAG-dependent signals, but probably to regulate PA generation. Given the relevance of DAG-dependent signalling and DGK expression in mammalian nervous and haemopoietic systems, in the next section we will review the more recent discoveries in these two systems.

DGK IN IMMUNITY AND INFLAMMATION

DGK α negatively regulates T-cell activation

Antigen-mediated triggering of the TCR (T-cell receptor) at the T-lymphocyte surface initiates a complex signalling mechanism

that, through tyrosine phosphorylation of different scaffold proteins, leads to PLC γ 1 activation. PLC γ 1-derived DAG is critical for the localization and activation of several proteins, including PKC α and θ , PKD and RasGRP1 [102].

Whereas defective T-cell activation results in a deficient immune response, excess activation gives rise to lymphoproliferative disorders. Following TCR triggering, the activation of negative regulatory pathways guarantees adequate control of signal intensity and duration. Part of this negative regulation is carried out by distinct tyrosine phosphatases, whereas additional control mechanisms rely on the termination of DAG-derived signals. T-lymphocytes express high DGK α and ζ levels, and both isoforms are proposed to exert a negative function in TCR response regulation. Most studies have used the well-known Jurkat T-cell model, in which TCR signalling can be elicited using antibodies against TCR complex components (i.e. anti-CD3), alone or in combination with antibodies against co-stimulatory molecules (i.e. anti-CD28) [53,103]. A Jurkat T-cell line stably transfected with the M $_1$ R (human muscarinic type 1 receptor), whose stimulation mimics TCR-triggered responses, has also been used to investigate DGK functions [53,64]. Generation of mice deficient in these isoforms has confirmed the results in cell lines, providing valuable information about the function of these enzymes [104,105].

Studies with GFP-fused chimaeras demonstrated that, in Jurkat T-cells, DGK α translocates to the membrane in a rapid transient manner following DAG production either by TCR triggering or by the stimulation of the M $_1$ R [53]. Translocation kinetics were more sustained for a kinase-defective mutant, suggesting that enzyme activity regulates its stability at the membrane. This study, the first to suggest a role for DGK α as a negative regulator of TCR signalling, also characterized the importance of the N-terminal

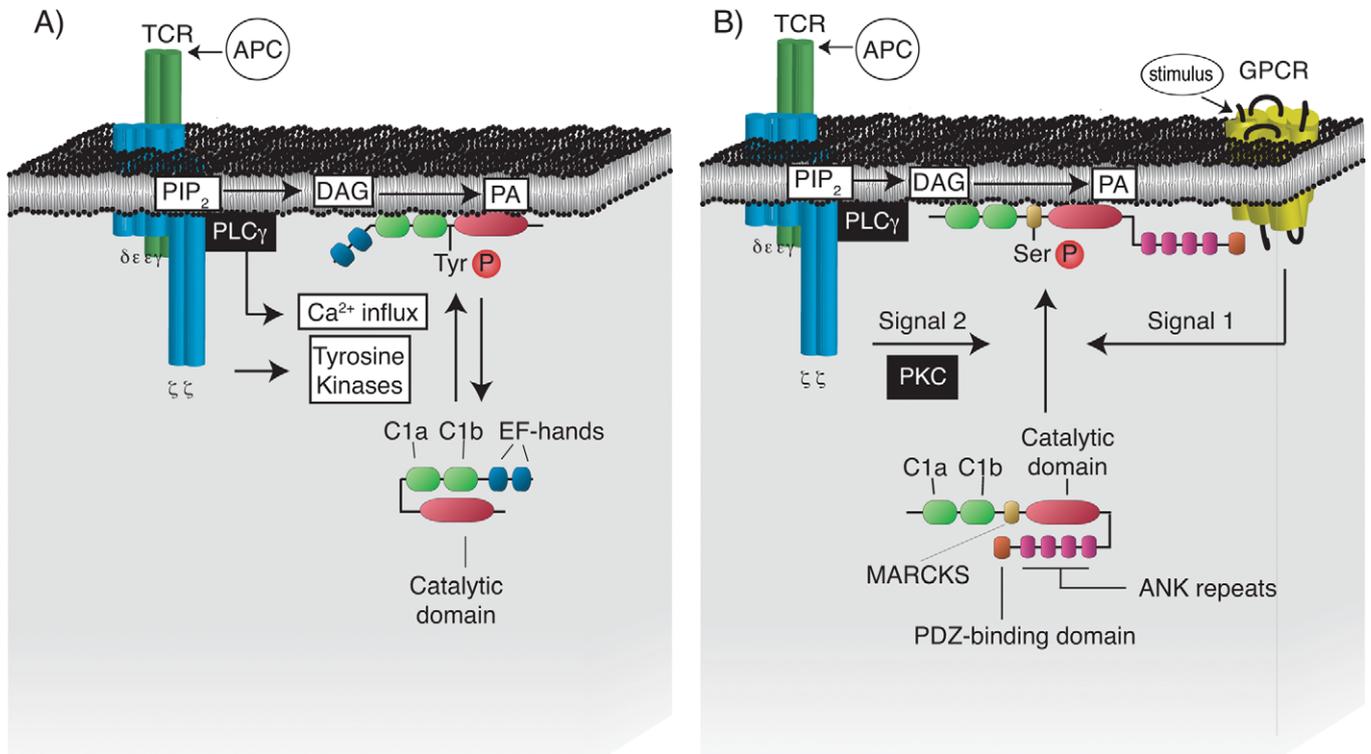


Figure 4 DGK α and DGK ζ are regulated by distinct mechanisms in response to TCR activation

TCR-dependent activation of tyrosine kinases activates PLC γ 1. Through PtdIns(4,5) P_2 (PIP $_2$) degradation, this enzyme generates DAG as well as Ins P_3 (IP $_3$), which increases intracellular Ca $^{2+}$. (A) DGK α translocation to the membrane is a rapid transient event regulated by its own activity. Ca $^{2+}$ elevation induces a conformational change that releases the negative regulation exerted by the DGK α N-terminal domain. Tyrosine phosphorylation induces an open active DGK α conformation that favours its membrane localization, where it phosphorylates DAG. DGK α -dependent PA production, together with dephosphorylation, restores the closed conformation and promotes translocation back to cytosol. (B) Sustained translocation of DGK ζ to the membrane requires a first GPCR-derived signal. This relieves the negative regulatory function of the DGK ζ C-terminal domain, favouring PKC-dependent phosphorylation at the MARCKS sequence; this promotes DGK ζ membrane recruitment and termination of DAG-derived signals. APC, antigen-presenting cell. An animated version of this Figure can be seen at <http://www.BiochemJ.org/bj/409/0001/bj4090001add.htm>.

regulatory domain in modulating enzyme translocation. The N-terminal region of the protein, encompassing the recoverin-like and the two EF-hand domains exerts a negative regulatory role, as shown by constitutive plasma membrane localization of a mutant lacking this region [53]. This constitutive localization and the sustained translocation of kinase-dead DGK α suggest that PA generation promotes enzyme dissociation from the membrane by acting on the regulatory N-terminal region. A model was thus proposed in which a conformational change elicited by Ca $^{2+}$ binding to the DGK α N-terminal domain regulatory regions would bring about membrane localization, whereas PA, also acting through this domain, would be responsible for membrane dissociation. Although necessary, Ca $^{2+}$ elevation is not sufficient to promote membrane translocation, and recent experiments suggest that Lck-dependent phosphorylation of a tyrosine at the hinge between the catalytic region and the C1 domain is critical for DGK α translocation to the membrane (E. Merino, A. Ávila-Flores, Y. Shirai, I. Moraga, N. Saito and I. Mérida, unpublished work) (Figure 4A).

DGK α translocation to the plasma membrane correlates with elevation of its enzyme activity. Studies in Jurkat T-cells showed that DGK α membrane localization and activation acts as a 'switch-off' signal for Ras activation, mediated by localization to the membrane of RasGRP1 [85,106]. These findings were confirmed *in vivo* by examining the kinetics of RasGRP1 and DGK α translocation during T-cell activation [85]. DGK α also acts as a negative regulator of PKC θ membrane localization in response to T-cell activation [107].

The generation of DGK α -deficient mice confirmed the results obtained in cell lines, showing that stimulation of DGK α -null T-cells elicits increased RasGTP levels and MAPK (mitogen-activated protein kinase) activation. Compared with their wild-type counterparts, T-cells from DGK α -deficient mice produce more IL (interleukin)-2 and show increased proliferation in response to TCR triggering [104]. Together, these studies indicate that DGK α controls the magnitude of the TCR response, acting as a brake at the initial steps of TCR signalling.

DGK α is an anergy-induced gene

Negative regulation of TCR-dependent signalling by DGK α affects not only the efficiency of the primary T-cell response, but also related processes, such as self-recognition, immune tolerance and autoimmune responses. One proposed peripheral tolerance mechanism is clonal anergy, a hyporesponsive state resulting from TCR engagement in the absence of co-stimulatory signals [108]. Studies in T-cells demonstrated efficient down-regulation of DGK α expression during T-cell activation [85]. DGK α protein is highly expressed in unstimulated cells, but mRNA levels decrease sharply after TCR activation. As a result, DGK α protein levels are lower in cycling T-cells than in resting T-cells [85]. These results correlate with the identification of DGK α as an anergy-induced gene [109], since a hallmark of anergic cells is their blockade in the G $_1$ -phase of the cell cycle that renders them unable to proliferate unless rescued by exogenous IL-2 [110] (Figure 5). Unresponsiveness in anergic cells has

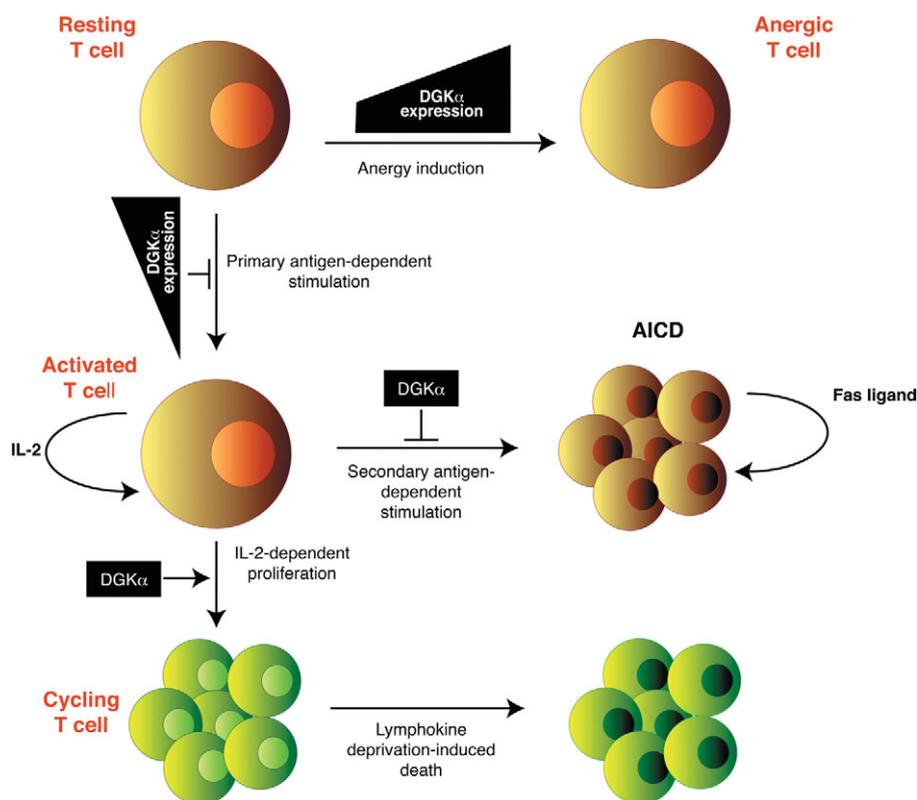


Figure 5 DGK α acts at different stages of T-cell activation and proliferation

Resting T-cells express high DGK α levels, which contribute to negative regulation of DAG-dependent signals during T-cell activation. DGK α expression decreases throughout T-cell activation, although its function is required for IL-2-dependent cell-cycle entry. Sustained DGK α elevation prevents full T-cell activation, leading to anergy. DGK α is also present in activated T-cells, where it regulates exosome secretion and AICD.

been linked to several events, among them reduced activation of the Ras/MAPK pathway. Remarkably, DGK α -deficient mice are refractory to anergy induction [104], and primary T-cells overexpressing DGK α resemble anergic cells, with reduced RasGRP recruitment to the immunological synapsis [111].

DGK α is a positive regulator of T-cell proliferation

Signalling through the TCR and its co-stimulatory proteins is essential for completion of the T-cell activation program. Once activated, the T-cell population expands through IL-2-dependent binding to its high-affinity receptor, which is expressed on the surface of activated T-cells. T-cell proliferation correlates with elevation of cellular PA as a result of IL-2-dependent DGK α activation [75]. In contrast with its negative role in the regulation of TCR-dependent responses through phosphorylation of PLC-derived DAG, DGK α -dependent PA generation by phosphorylation of a pre-existing DAG pool is necessary for IL-2-dependent proliferation [112]. IL-2 is a cytokine that activates the JAK (Janus kinase)/STAT (signal transducer and activator of transcription) pathway independently of PLC γ activation, suggesting DGK α participation as a positive regulator in this route (Figure 5). This positive role in JAK/STAT regulation is consistent with recent studies showing that DGK α is essential for proliferation and/or survival of ALCL (anaplastic large cell lymphoma) [113]. The negative role of DGK α in TCR regulation precludes study of its contribution to IL-2-dependent proliferation in DGK α -deficient mice; these studies will only be feasible when conditional knockouts for this isoform become available.

DGK α regulates AICD (activation-induced cell death)

A properly functioning immune response requires the phenomenon known as AICD, in which activation through the TCR results in apoptosis [114]. In peripheral T-cells, AICD results from the interaction between Fas and Fas ligand and is essential for maintaining tolerance to self-antigens. Defects in AICD are linked to the development of autoimmunity [115]. DGK α regulates secretion of FasL-bearing exosomes during AICD, through a mechanism that apparently relies on a reduction in the DAG levels needed for vesicle fusion and transport from the *trans*-Golgi network [116]. This suggests distinct DGK α functions depending on its subcellular localization. Whereas down-regulation of early TCR responses depends on DGK α plasma membrane localization, regulation of exosome secretion requires enzyme association to internal membrane compartments (Figure 5).

DGK ζ provides additional mechanisms to control T-cell activation

In addition to DGK α , T-lymphocytes also express DGK ζ , for which a negative regulatory function has also been proposed. DGK ζ overexpression in a Jurkat T-cell variant clone (J14) decreases Ras loading and MAPK activation, as well as AP-1 (activator protein 1) induction and CD69 expression, in response to TCR stimulation [103]. Although the function of these two DGK isoforms appears to be similar, studies in GFP-coupled protein chimaeras suggests that the mechanisms by which each isoform localizes to the membrane are quite different. In Jurkat T-cells, GFP-DGK α translocates rapidly and transiently in response to both endogenous TCR and ectopically expressed M $_1$ R stimulation

[53]. GFP-DGK ζ translocation is rapid and sustained in response to M $_1$ R by a mechanism dependent on PKC activation [64]. No translocation of this isoform is observed in response to TCR stimulation, suggesting specific regulation of this isoform by GPCRs (G-protein-coupled receptors). The DGK ζ C-terminal region, which encompasses several ankyrin repeats and a PDZ-domain-binding motif, has a restrictive regulatory function, providing GPCR-dependent specificity. Translocation was TCR-dependent in a truncated mutant lacking this region, indicating that DGK ζ translocation requires of two consecutive signals, the first of which, coupled to G-protein, circumvents the negative regulation imposed by this region [64]. These results strongly suggest that DGK ζ modulation of TCR responses must be conditioned by the co-stimulatory context. DGK were recently shown to form complexes with arrestins that, after stimulation, associate with GPCRs [69]; this points to the possibility that GPCR-derived co-stimulatory signals might prime DGK ζ for membrane translocation in response to TCR triggering. This hypothesis is reinforced by recent results showing active cross-talk between TCR and GPCR [117] (Figure 4B).

The negative function of DGK ζ is confirmed by the phenotype of DGK ζ -deficient mice, which show Ras/MAPK activation, as well as enhanced expression of the CD69 and CD25 activation markers [105]. Overexpression experiments showed that DGK ζ binds to RasGRP1 in non-haemopoietic cells [86]. Differently from DGK α , there is no evidence for a negative effect of this isoform on RasGRP1 translocation to membrane. Nevertheless, CD4 $^+$ T-cells derived from DGK ζ -null mice are also anergy-resistant and produce even more IL-2 than DGK α -deficient cells [104]. Accordingly, DGK ζ protein levels are reported to increase, although less than those of DGK α , during anergy induction [111].

DGK in thymic selection

In addition to its essential function for TCR activation in the periphery, DAG has a key role in the thymus. Thymic selection depends on TCR-derived signal intensity: strong TCR activation leads to thymocyte apoptosis through negative selection, whereas intermediate stimulation drives cells to differentiate into mature T-cells through positive selection [118]. The intensity of the Ras/MAPK signalling cascade is thought to be essential in positive selection [119]. This intensity is largely driven by the subcellular localization of active Ras; localization at the plasma membrane triggers strong signals and at internal membranes elicits weak signals [120]. According to this model, ligand affinity would modulate DGK activation, leading to appropriate cell fate in the thymus. DGK α is regulated during thymic differentiation, and its activity modulates survival of double-negative thymocytes in foetal thymic organ cultures [121]. DGK α -deficient mice nonetheless have normal thymocyte numbers and populations [104], as is also the case for DGK ζ -deficient animals [105]. Dramatic defects in thymocyte development are nevertheless encountered in mice lacking both isoforms [104]. These mice have few CD4 $^+$ and CD8 $^+$ thymocytes, and peripheral T-cells are inappropriately inactivated, suggesting important DGK functions in the control of thymic development.

These studies indicate that the acquired immune response is highly dependent on DGK activity, and suggest that, although DGK α and DGK ζ show some functional redundancy, they have specific non-redundant roles in the control of TCR responses. Accordingly, siRNA (small interfering RNA) knockdown of these isoforms in Jurkat T-cells demonstrates well-differentiated functions for each. DGK α acts at the initiation of the TCR signalling cascade, controlling the magnitude of the response; its effects are nonetheless silenced by co-stimulation. The main

function of DGK ζ is to turn off the activation response, and its recruitment to the TCR signalling complex is strongly influenced by GPCR-dependent co-stimulation (A. Ávila-Flores, E. Merino and I. Mérida, unpublished work).

DGK ζ in innate immunity

DGK ζ is expressed in dendritic cells and macrophages, and levels increase after LPS (lipopolysaccharide) stimulation. Analysis of DGK ζ -deficient mice suggests that it is a positive modulator of TLR (Toll-like receptor) signalling. These mice show deficient IL-12 and TNF α (tumour necrosis factor α) production after TLR stimulation, are resistant to endotoxic shock and are more susceptible to *Toxoplasma gondii* infection than controls [122]. Although the underlying mechanism was not demonstrated, DGK ζ deficiency induced minor increases in ERK (extracellular-signal-regulated kinase) phosphorylation and I κ B [inhibitor of NF- κ B (nuclear factor κ B)] degradation, and greatly augmented the PI3K/Akt pathway. The observed effects are proposed to be mediated through DGK ζ -dependent negative regulation of the PI3K/Akt pathway, which is thought to negatively regulate TLR signalling. DGK ζ deficiency-mediated effects were reverted by exogenous PA addition, suggesting that the main function of DGK ζ in this model is not to diminish DAG, but to elevate PA. The role of DGK-derived PA in the PI3K/Akt pathway remains undetermined. It is remarkable that these mice show a more robust immune response to choriomeningitis virus infection, which is also TLR-mediated [105]. This suggests that DGK ζ may differentially affect the signalling elicited by different TLRs.

DGK and inflammatory responses

Mast cells initiate allergic responses through the release of histamine-containing granules and by secreting inflammatory cytokines. Fc ϵ RI (high-affinity receptor for IgE), which is critical for mast cell function, induces a signalling cascade in which PLC γ is activated, as are the MAPK and PI3K pathways. Studies in DGK ζ -deficient mice show a critical role for this isoform in the regulation of Fc ϵ RI-mediated signals. DGK ζ -deficient bone-marrow-derived mast cells show decreased PLC γ activation after Fc ϵ RI cross-linking, probably reflecting a role for DGK ζ -derived PA in PtdIns synthesis. Defective PLC γ activation in DGK ζ $^{-/-}$ mast cells results in defective PKC β_{II} membrane recruitment that correlates with impaired degranulation [104]. As a consequence these mice show impaired anaphylactic responses. Notably, RasGRP1-deficient mice also fail to mount allergic reactions [122]. RasGRP1 $^{-/-}$ mast cells show reduced degranulation, suggesting that impaired PLC γ activation affects RasGRP1 regulation. Interestingly DGK ζ deletion enhances Ras/ERK signals and IL-6 production, suggesting an additional role for DGK activity for adequate attenuation of Fc ϵ RI signals [123]. These studies show an interesting dissociation between cytokine production and degranulation in mast cells, revealing an important, although contradictory, role for DGK ζ in the regulation of these processes.

Defective DGK α function is linked to LAP (localized aggressive periodontitis), a genetic disorder characterized by destruction of the supporting structures for dentition. Some reports link the disease to defects in neutrophil function, whereas others propose that LAP is the result of chronic neutrophil hyperactivation [124]. A recent study correlated LAP-linked decreases in neutrophil transmigration and increases in superoxide generation with reduced DGK α expression and activity [125]. Since superoxide production relies on the phosphorylation of p47 phox protein by a DAG-sensitive PKC [126], DGK α

probably controls superoxide production through PKC down-regulation. Additional functions for DGK have been reported recently by showing selective activation of this enzyme by anti-neutrophil cytoplasmic antibodies in patients with certain forms of systematic vasculitis such as Wegener's granulomatosis [127]. In this case, the DGK-dependent PA generation, triggered by antibody binding, is responsible for promoting abnormal neutrophil adhesion that results in obstruction of small vessels [127].

DGK FUNCTION IN THE NERVOUS SYSTEM

DGK family members are highly expressed in the brain, suggesting an important role for this lipid kinase family in the nervous system. DAG activates a characteristic type of Ca^{2+} channel, as well as DAG receptors such as PKC or Munc family members. DAG and PA are also involved in the traffic and fusion of synaptic vesicles. In addition to controlling the levels of these two lipids, DGK might have a scaffold role, recruiting proteins needed for events such as dendritic establishment or axonal guidance.

Lessons from worms and flies

C. elegans and *D. melanogaster* have provided a genetically tractable model for elucidating the physiological roles of certain DGKs in the nervous system. *C. elegans* DGK-1 is expressed in neurons and is 40% identical with human DGK θ [84]. Genetic studies show that DGK-1 modulates the DAG generated by $\text{G}\alpha_q$ signalling in response to neurotransmitters. DAG depletion by DGK-1 modulates the function of the synaptic vesicle priming protein UNC13, a C1-domain-containing protein that requires DAG binding to promote neurotransmission. Loss of DGK-1 leads to increased UNC13-mediated neurotransmission and, as a consequence, to strong behavioural defects, including altered dopamine-controlled locomotion and serotonin-controlled egg laying [83,84]. A similar alteration is observed after loss of $\text{G}\alpha_o$, due to an increase in UNC13 at synaptic release sites. The genetic data allowed the proposal of a model in which DAG accumulation is the result of $\text{G}\alpha_q$ -mediated PLC β activation, whereas the $\text{G}\alpha_o$ protein GOA-1 activates DGK-1, which depletes DAG levels [128]. Additional regulatory mechanisms are provided by the Rho orthologue RHO-1 that binds to DGK-1, promoting its inactivation. This results in DAG elevation and consequent UNC13 accumulation at neurotransmitter-release sites [68] (Figure 6).

In addition to synaptic transmission, DAG controls processes such as olfactory adaptation. In this case, the lack of olfactory adaptation observed in GOA-1 mutants is not mimicked by DGK-1 loss of function, suggesting that different DGKs are implicated in this process. Neither individual DGK-2 (DGK ϵ orthologue) nor DGK-3 (Type I DGK orthologue) loss of function results in loss of adaptation, which is observed, however, in the double DGK-1/DGK-3 mutant. This suggests functional redundancy of certain DGK isoforms for certain specific functions [129]. Further enlarging the complex role of DGK in the regulation of *C. elegans* sensing and behaviour, DGK-3 was recently identified as a thermal memory molecule that governs the temperature range of synaptic output [130]. DGK-3 is highly expressed in AFD neurons, and nematode worms with mutations in this enzyme show slower resetting of their culture temperature. Thermal resetting defects can be mimicked using DAG analogues, whereas overexpression of EF-hand-deleted DGK-3 mutants induces more rapid thermal resetting. Temperature changes induce elevation in intracellular Ca^{2+} in AFD neurons, leading to DGK-3 activation. The thermo-DAG targets in AFD neurons are not known, but circuits are likely to resemble those described for the other two *C. elegans* DGKs.

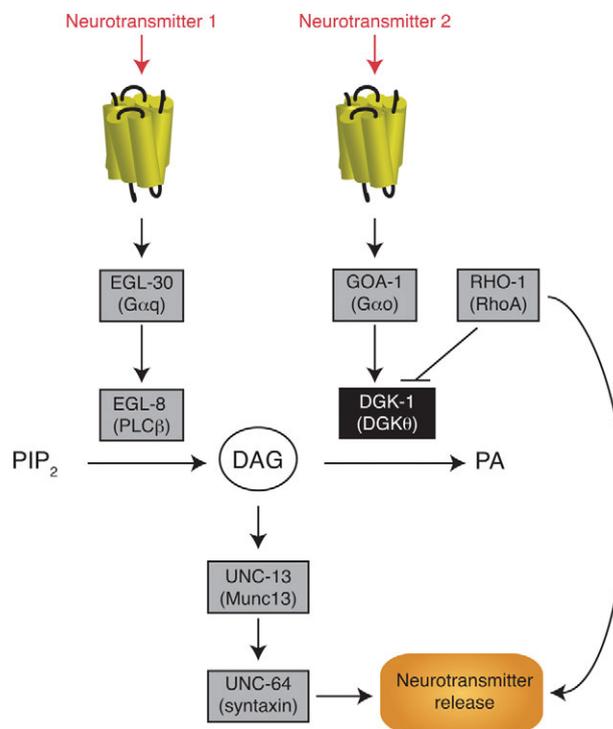


Figure 6 DGK-1 function in *C. elegans*

UNC13 accumulation at neurotransmitter-release sites is induced by DAG elevation as the result of EGL-30-mediated EGL-8 activation. The $\text{G}\alpha_o$ protein GOA-1 activates DGK-1, which depletes DAG levels; RHO-1 binds to DGK-1, promoting its inactivation. Modified from McMullan, R., Hiley, E., Morrison, P. and Nurrish, S.J. (2006) Rho is a presynaptic activator of neurotransmitter release at pre-existing synapses in *C. elegans*. *Genes Dev.* **20**, 65–76, with permission. © 2006 Cold Spring Harbor Laboratory Press.

Studies of *C. elegans* provide one of the clearest illustrations of the critical role that localized DAG generation has in the regulation of synaptic vesicle dynamics. UNC13 function is highly conserved in the stage following vesicle docking and before fusion, as shown by elimination of *Dunc13*, the *Drosophila* homologue [131]. In mammals, the four Munc13 proteins are critical for synaptic transmission, insulin secretion and cytotoxic responses, suggesting that this protein family provides a critical link between DAG generation and the regulation of polarized responses [132–134]. The DAG requirement for Munc13 function was elegantly demonstrated by showing lack of potentiation of the synaptic response in knockin mice bearing a Munc13-1 mutant unable to bind DAG [135]. In mammals, DAG apparently has a more complex role in regulating synaptic transmission, as shown by recent experiments demonstrating that PKC co-operates with Munc13 to potentiate transmission [136]. That study illustrates the role of DAG acting on two targets in close succession to potentiate signalling. To date, no studies have addressed the role of DGK in Munc13 protein regulation, but it is reasonable to expect that DGKs will act as negative regulators of polarized secretion in nervous and immune synapses.

Genetic analysis of *Drosophila* retinal degeneration mutants characterized the importance of DGK in this mechanism. In *Drosophila* photoreceptor cells, light-dependent isomerization of rhodopsin initiates a classical G-protein transduction cascade; the $\text{G}\alpha_q$ subunit activates PLC (*norpA*), which forms a signalosome complex in which the multi-PDZ-domain-containing protein INAD (inactivation no afterpotential D) maintains PLC in close proximity to PKC (*inacC*). This allows efficient activation of

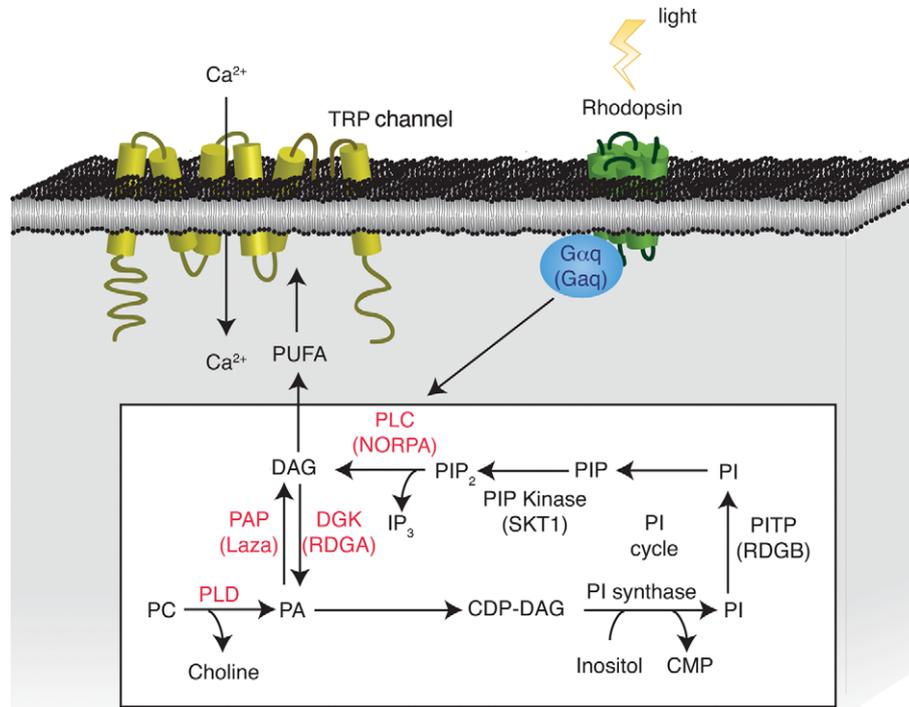


Figure 7 DGK function in *Drosophila* photoreceptor cells

Light-dependent isomerization of rhodopsin initiates a classical G-protein transduction cascade; $G\alpha_q$ -mediated NORPA activation allows efficient TRP channel activation. Either directly or through polyunsaturated fatty acids (PUFA), DAG is indispensable for this function. RDGA regulates DAG conversion into PA, which feeds into the PtdIns (PI) cycle and is the substrate for *Laza* activity; this catalyses its conversion into DAG. PLD activation provides an alternative PA source for *Laza*-mediated DAG production. IP_3 , $InsP_3$; PAP, PA phosphohydrolase; PC, phosphatidylcholine; PIP, PtdInsP; PIP_2 , PtdIns P_2 ; PITP, phosphatidylinositol-transfer protein.

the TRP (transient receptor potential) channel, which mediates Ca^{2+} entry into the cell (Figure 7). Although the exact mechanism is a matter of debate, it is generally accepted that PLC-dependent DAG generation is indispensable for TRP channel activation. Moreover, DAG seems to have a dual function, one stimulatory and PKC-independent, and the other inhibitory and PKC-dependent [137].

Mutation in the *rdgA* gene, which encodes the *Drosophila* DGK2 homologue of mammalian DGK ι , is the most severe of the many causes of *Drosophila* retinal degeneration. *rdgA*-induced degeneration is light-independent, and photoreceptors are destroyed in newborn flies. Photoreceptors in these mutants show constitutive TRP channel activity and defects in response termination; these defects are rescued in *rdgA-norPA* and *rdgA-Gaq* double mutants [138,139]. This strongly suggests a role for DAG as an excitatory messenger, which is confirmed further by mutations in related components of the PtdIns(4,5) P_2 /DAG pathway [140], although some data appear to contradict this assumption. *Drosophila* TRP channels do not have a canonical DAG-binding region; although this might not exclude indirect activation, no DAG elevation is observed in *rdgA* mutants, and exogenous DAG does not stimulate *Drosophila* TRP channels *in vitro* [139].

Whereas experimental evidence pointed to DGK as an essential brake on the magnitude of TRP channel activation, the mechanism by which *rdgA* mutations lead to retinal degeneration long remained obscure. Two recent reports identifying PAP (PA phosphohydrolase) Lazaro (*Laza*) provided important clues regarding the molecular basis for retinal degeneration in these mutants [141,142]. The severity of the *rdgA* phenotype is reverted in *Laza* loss-of-function mutants, demonstrating that these two

enzymes act in opposite directions. These studies suggest that depletion of PA, essential for generation of PtdIns and its metabolites, is the key signal for retinal degeneration. PA involvement in phototransduction is heightened by the retinal degeneration observed in PLD (phospholipase D)-null flies (*Pld*^{-/-}) [143]. Together, these studies suggest that *rdgA*-mediated PA generation facilitates phototransduction through a number of critical functions. PA is a substrate for Lazaro, which catalyses its conversion into DAG, sustaining TRP channel activation; PA feeds into CDP-DAG to replenish PtdIns(4,5) P_2 levels; it also regulates transcription of PI synthase and at the same time acts as an allosteric regulator of type I PIPkin [141]. The genetic interaction of *Laza* with both *rdgA* and *pld* indicates that *in vivo*, this phosphatase functions at the convergence of the PLC and PLD pathways, and provides a role for PLD in DAG production (Figure 7).

The *Drosophila* retina thus provides an excellent model to exemplify the complex role of DGK, not only as a key regulator of DAG levels, but also as a pivotal component of the PtdIns cycle. Through PA production, DGK regulates PLC substrate levels as well as activation of the enzymes that produce it. Confirming this role, *rdgA* mutants do not have elevated DAG levels; on the contrary, they show decreased PA levels and PtdIns synthesis, probably responsible for impaired DAG accumulation [141]. This intimate correlation between DGK activity and PtdIns levels is probably inherent to all DGKs, and may explain observations made in other DGK-deficiency models such as DGK ζ - and DGK ϵ -null mice.

Drosophila TRP channels are similar to the mammalian TRPCs (canonical TRP channels), a broadly expressed seven-member family. All TRP channels are activated by PLC-dependent

mechanisms, but only TRPC 2, 3, 6 and 7 are activated by DAG. Many of these channels are linked to important physiological processes as well as to some pathological alterations [144]. The role of DGK in the regulation of mammalian TRPCs has yet to be examined.

DGK in the mammalian nervous system: a world to discover

Many reports suggest diverse functions for DGK in the regulation of the nervous system. The DGK α isoform is detected in oligodendrocytes [145], the myelin-producer cells; this enzyme activity was in fact proposed to be an intrinsic myelin component [146]. IL-2-dependent DGK α activation, as described in T-lymphocytes [147], has been proposed to participate in myelin regeneration [148]. Myelin forms specialized sheaths that insulate axons and facilitates action potential propagation by saltatory conduction. Successful axon regeneration in the mammalian central nervous system is compromised, at least in part, by inhibitors associated with myelin and glial scar. There is evidence that conventional PKC isoenzymes are key components in the signalling pathway that mediates the inhibitory activities of myelin components [149]. Although it has not been studied formally, it would be interesting to examine the potential of DGK α as a PKC-inhibitory agent.

Although it is not brain-specific, DGK β has a unique expression pattern in rat brain areas that are known to be important in synaptic transmission of cognitive and emotional processes in the central nervous system [150]. In humans, the DGK β gene can generate as many as 16 isoforms [30]. Of particular interest are the splice variants that display a 35-amino-acid C-terminal deletion, since bipolar disorder patients differentially express a similar mRNA. When overexpressed in COS cells, full-length DGK β translocates to the membrane in a phorbol ester-dependent manner; translocation of the truncated version is impaired even in the presence of the phorbol ester-binding C1 domain [30]. How the imbalanced expression of truncated DGK β contributes to the aetiology and/or pathology of mood disorders it is not known. Owing to their ineffective membrane recruitment, these variants might cause incorrect attenuation of the DAG generated after receptor stimulation. Another DGK, in this case the η isoform, was also recently linked to the aetiology of bipolar disorder [151].

The DGK γ isoform is expressed highly in retina and brain, mainly in cerebellar Purkinje cells, and is predominantly cytoskeleton-associated [26]. Most tissues other than retina and brain express a truncated, and thus inactive, DGK γ form [27]. A functional correlation is suggested between DGK γ and PKC γ , which is also highly expressed in Purkinje cells. Purinergic receptor-dependent elevation of Ca²⁺ and DAG targets PKC γ to the plasma membrane [66]. DGK γ also moves to the plasma membrane, although its kinetics are slower because of distinct Ca²⁺ sensitivity. Once at the membrane, DGK γ binds PKC γ , which phosphorylates the DGK γ accessory domain. This activates DGK γ , leading to DAG consumption, which causes PKC γ redistribution to the cytoplasm and attenuation of its activity. DGK γ remains at the membrane, perhaps through interaction with other proteins or lipids, and then returns to the cytosol [81].

DGK ϵ is the only isoform with *in vitro* substrate specificity for arachidonoyldiacylglycerol (C_{20:4}-DAG). Although broadly expressed, DGK ϵ is very abundant in retina and brain [152,153]. A role has been suggested for DGK ϵ in the regulation of mGluR (metabotropic glutamate receptor)-dependent signalling. DGK ϵ -deficient mice present no obvious histological alteration in brain, although they are resistant to electroconvulsive shock. This resistance seems to be due to diminished mGluR-dependent

activation of the PtdIns cycle, since mice do not show DAG accumulation [153]. This reduction in PtdIns recycling parallels that observed in *rdgA* mutants, suggesting a similar role for DGK ϵ as a PtdIns cycle component. These data also suggest that DGK ϵ -controlled lipids are important in the genesis of epilepsy. It has been shown that lack of DGK ϵ isoform also has an effect on epilepsy-induced genes such as COX-2 (cyclo-oxygenase 2) and tyrosine hydroxylase in the hippocampus [154,155]. Since this isoform is highly expressed in the retina, a possible link has also been suggested with inherited retinitis pigmentosa, although a preliminary study did not reveal DGK ϵ gene mutations [156].

DGK ζ is abundantly expressed in specific brain regions and is strictly regulated during development [157,158]. Several functions have been proposed for this isoform. DGK ζ is cytosolic; it forms a complex with RacGDP and syntrophins, and regulates neurite formation by directing translocation of this complex to growth cones and sites of early process formation [100]. The enhancer role of DGK ζ in neurite growth relies on its scaffolding function, which promotes Rac localization at the plasma membrane, independently of DGK ζ catalytic activity. Neurons also express high levels of DGK γ , which is reported to act as an upstream suppressor of Rac1 [159]. In contrast with DGK ζ , DGK γ activity inhibits Rac activation, but no interaction between these two proteins has been reported. This suppressive effect on Rac activation could be mediated by DGK γ -dependent activation of β 2 chimaerin, a GAP for Rac that interacts with this DGK isoform [88].

DGK ζ is present in the hippocampus, a region highly sensitive to hypoxia. Under normal conditions, hippocampal neurons show immunoreactivity for nuclear DGK ζ ; during the early phase of ischaemia, DGK ζ exits rapidly from the nucleus. Reperfusion treatment does not reverse this translocation, but reduces cytoplasmic DGK ζ levels [160]. DGK ζ regulation during ischaemia contrasts with that observed for DGK ι , another type IV DGK that also has an NLS, but whose cytoplasmic distribution is unaltered [161].

The brain requires a continuous oxygen and nutrient supply to maintain normal function. Acute insults such as hypoxia and ischaemia elevate levels of Ca²⁺ and DAG, which are probably responsible for the high PKC activity observed during early ischaemia. The function of nucleocytoplasmic DGK ζ translocation in early ischaemia remains to be determined; it may induce sustained PKC activation in the nucleus, which is thought to cause neuron damage. In addition to its rapid effects on cell signalling, hypoxia also modulates transcriptional responses through HIF (hypoxia-inducible factor) 1 α . Studies in non-neuronal cells show that acute hypoxia correlates with elevated DAG and PA levels, and that HIF1 stabilization requires DGK activity [162]. Further studies are needed to determine the contribution of DGK in cell adaptation to hypoxia compared with hypoxia-induced cell damage.

The characterization of DGK ζ as a component of the leptin signalling pathway suggests another function for this isoform in brain. Leptin regulates food intake and body weight through activation of its long-form Ob-Rb receptor on hypothalamic neurons. Mutations in the hormone or its receptor lead to morbid obesity [163]. Via its ankyrin repeats, DGK ζ binds to the Ob-Rb cytoplasmic tail. A high-fat diet together with leptin production increases hypothalamic DGK ζ mRNA levels [164]. That study shows that DGK ζ activity is regulated by its interaction with a leptin-stimulated Ob-Rb, and that leptin or Ob-Rb mutations lead to decreased DGK ζ activity, despite elevated DGK ζ mRNA levels in hypothalamus. DGK ζ may thus participate in the control of body fat accumulation by directly regulating DAG levels in the synthesis of complex lipids.

DGK AND HUMAN DISEASE

In addition to these major roles in neurons and haemopoietic tissues, DGK-dependent modulation of DAG levels represents a major checkpoint in other systems. Several pathological states lead to excess DAG production, which may be modulated by DGK activity. The next sections illustrate the impact of DGK regulation in the control of certain diseases.

DGK and diabetes

High glucose levels, characteristic of all forms of diabetes, are responsible for the development of diabetes-specific microvascular pathologies in the retina, renal glomerulus and peripheral nerves; as a result, diabetes is a leading cause of blindness, renal disease and nerve damage. Intracellular hyperglycaemia promotes abnormal DAG accumulation, not from PLC activation, but from *de novo* synthesis [165]. Increased *de novo* DAG synthesis induces activation of several PKC isoforms, particularly PKC β [166]. Deregulated PKC activation has a number of pathogenic consequences that affect expression of eNOS (endothelial nitric oxide synthetase), endothelin-1, VEGF (vascular endothelial growth factor) and PAI-1 (plasminogen activator inhibitor-1). Several recent studies have demonstrated that some of the agents used to counteract hyperglycaemia and control diabetes may act through regulation of DGK levels and/or activity (Figure 8A). Compounds such as D- α -tocopherol or vitamin E, which have a common chromene ring structure, can prevent hyperglycaemia-induced DAG elevation and PKC activation. In addition to their main antioxidant role, these compounds are proposed to promote DGK α translocation to the plasma membrane [167]. The means by which these compounds promote enzyme translocation has not been fully determined, although experimental evidence points to tyrosine kinase-mediated DGK α phosphorylation as the mechanism that modulates membrane translocation [167].

Another important type of pharmacological agent used to treat diabetic complications are agonists of PPAR γ (peroxisome-proliferator-activated receptor γ), a ligand-activated transcription factor originally identified as a central modulator of adipogenesis and glucose homeostasis. PPAR γ is expressed in haemopoietic cells and endothelium [168]. PPAR γ agonists such as thiazolidinediones are suggested to have beneficial effects on the vasculature, and recent studies report that certain PPAR γ agonists transcriptionally up-regulate DGK α mRNA, resulting in suppression of the DAG–PKC axis [169]. Both mRNA and protein levels of DGK α , but not those of DGK β and DGK γ , were elevated following treatment of endothelial cells with PPAR γ agonists. Analysis of the DGK α gene sequence shows at least two PPAR-responsive elements in its genomic region, supporting further the idea that PPAR γ might down-modulate PKC activation by transcriptional regulation of DGK α .

DGK and heart disease

MI (myocardial infarction) induces LV (left ventricular) remodelling. Early changes in LV architecture after MI are compensatory phenomena to adapt and preserve cardiac performance. Progressive changes can nonetheless lead to congestive heart failure and increased mortality. Indeed, patients who escape death during the acute stage of MI are at risk of developing heart failure during the chronic stage. Although the molecular mechanisms of LV remodelling are not fully understood, PKC activation in response to GPCR agonists such as endothelin-1 and angiotensin have an important role in the development of cardiac hypertrophy and progression of heart failure [170]. Several studies suggest that DGK may be involved in regulating cardiac dysfunction.

In situ hybridization studies show that DGK ζ , DGK ϵ and DGK α are expressed in adult rat heart [171]. Whereas the functional significance of DGK ϵ and DGK α in cardiac hypertrophy remains to be clarified, reports suggest that DGK ζ attenuates LV remodelling after MI. Adenoviral expression of DGK ζ in cultured rat neonatal cardiomyocytes prevents endothelin-1-induced increases in protein synthesis and reactivation of foetal genes via inhibition of the PKC ϵ –ERK–AP-1 signalling pathway [172]. Generation of transgenic mice with cardiac-specific DGK ζ overexpression demonstrates that this enzyme prevents angiotensin II and phenylephrine-induced activation of PKC downstream signalling cascades and subsequent cardiac hypertrophy [173] (Figure 8B). In the same model, cardiac-specific DGK ζ overexpression blocks fibrosis in the non-infarct area, attenuates LV remodelling and improves post-MI survival [174]. In summary, these studies suggest that the DGK may represent novel therapeutic targets for the prevention of cardiac disease.

DGK and cancer

Sustained elevation of DAG levels is associated with malignant transformation, an observation largely supported by the potent tumour-promoter effect of the phorbol esters, which are synthetic DAG analogues. The negative regulatory function of DGK in DAG-mediated effects would suggest a suppressor role for this family in malignant transformation. However, and probably reflecting the complex roles of DAG-regulated molecules in cancer [175], DGKs are reported to act both as tumour suppressors and as positive regulators of survival and proliferation in transformed cells [176].

An example of this contradictory function is provided by DGK α , which, as is the case in immune response, appears to have a dual role in cancer progression. Studies in lung cancer that correlate gene-expression profiles with clinical outcome identified DGK α as a predictive marker of good prognosis [177], suggesting a potential tumour-suppressor role. In accordance, studies of astrocytes [178], human lung cancer cells [179] and mouse embryonic fibroblasts [180,181] characterize DGK α as a gene up-regulated by p53, a tumour suppressor that is mutated in most cancers.

On the other hand, studies of ALCLs indicate a positive role for DGK α in cell survival and proliferation [113]. The cell death induced after pharmacological inhibition or attenuation of DGK α expression by siRNA suggest an anti-apoptotic role for this isoform in these lymphomas. A similar role was also recognized in melanoma cells, where DGK α is highly expressed, as opposed to non-cancerous melanocytes, where is absent. In melanoma, reduction of DGK α protein levels inhibits TNF α -regulated NF- κ B activation [182]. DGK α -derived PA is necessary for VEGF- and HGF (hepatocyte growth factor)-induced migration and invasion in epithelial cells [183,184]. Recent studies have suggested that this isoform could be a pharmacological target for the treatment of some types of breast cancer [176].

Contradictory effects on proliferation are also reported for DGK ζ . In some cell types, the nuclear localization of this isoform correlates with accumulation in the G₁-phase of the cell cycle [54,74]. The negative effect of DGK ζ on cell-cycle progression is presumably related to the reduction in nuclear DAG levels, although the precise mechanism remains unknown. *In vitro* studies show that DGK ζ interacts with the hypophosphorylated pRb (retinoblastoma protein), a key tumour suppressor that controls S-phase entry, and that this interaction leads to increased DGK ζ activity [185]. There may be reciprocal regulation between these proteins, since DGK ζ overexpression in C2C12 myoblasts leads to pRb hypophosphorylation [74].

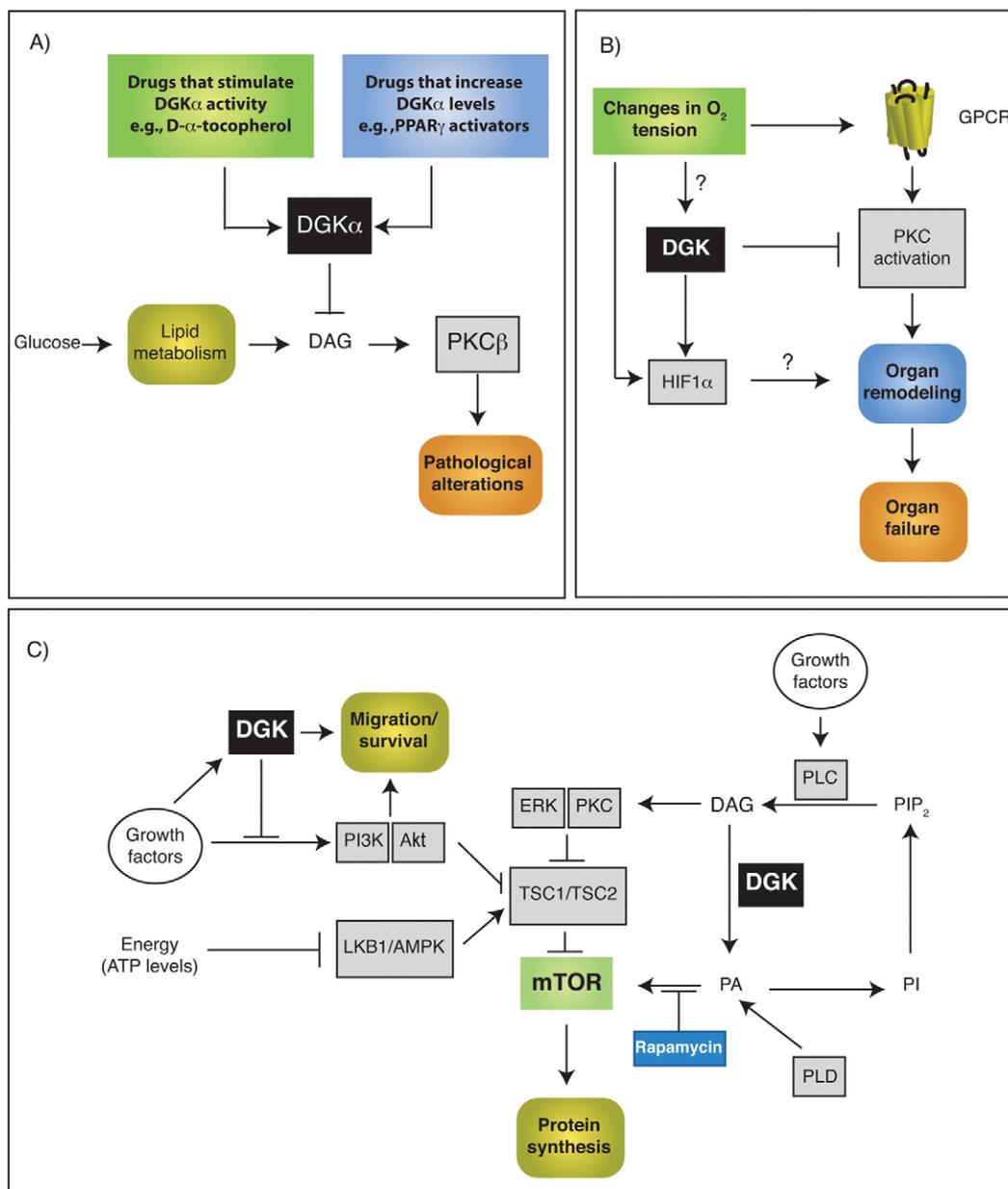


Figure 8 DGK function in pathological conditions

(A) DGK and diabetes. High glucose levels, characteristic of all forms of diabetes, promote abnormal *de novo* DAG accumulation; this results in deregulated PKC activation, with pathogenic consequences. Compounds such as D- α -tocopherol promote DGK α translocation to the plasma membrane, whereas PPAR γ agonists transcriptionally up-regulate DGK α mRNA. Modulation of DGK α translocation and/or expression levels lead to suppression of the pathological DAG–PKC axis. **(B)** DGK and heart disease. PKC activation in response to GPCR agonists such as endothelin-1 and angiotensin contributes to development of cardiac hypertrophy and progression of heart failure. Modulation of DGK levels and/or activity as a result of changes in O $_2$ tension might affect the regulation of cardiac dysfunction. **(C)** DGK and cancer. DGKs can act as both tumour suppressors and as positive regulators of growth factors, depending on isoform, cell type and/or the receptor involved. In epithelial cells, DGK α is implicated in VEGF- and HGF-induced migration and invasion. DGK ζ provides an alternative PA source to activate mTOR, counteracting rapamycin action. mTOR integrates several metabolic pathways by sensing oxygen and nutrient availability; DGK-dependent mTOR regulation provides a connection with lipid metabolism. Both DGK isoforms are reported to negatively regulate growth-factor-dependent activation of PKC/ERK and/or PI3K/Akt pathways. AMPK: AMP-activated protein kinase; PI, PtdIns; PIP $_2$, PtdInsP $_2$; TSC1/2: tuberous sclerosis complex 1/2.

Whereas DGK ζ negatively regulates cell proliferation as a result of attenuation of nuclear DAG levels, DGK ζ -produced PA positively regulates mTOR activity, hinting at a positive role for the isoenzyme in cell growth and survival [95]. Although PLD was proposed as the main source of the PA that regulates mTOR activity, DGK and LPA acyltransferases represent either alternative or PLD-interconnected sources of PA [186–188]. mTOR activation, mainly by PA derived from biosynthetic pathways, suggests a direct connection between mTOR activation

and phospholipid synthesis. mTOR is a master regulator that integrates several signalling pathways that sense oxygen and nutrient availability; PA-dependent regulation of mTOR would provide a somewhat expected connection with lipid metabolism (Figure 8C).

The complexity of the DGK role in the regulation of cell growth is illustrated further by the phenotype of certain DGK-null mice. Disruption of the DGK δ gene in mice reduces EGF (epidermal growth factor) receptor expression and activity, as

a result of increased PKC-dependent phosphorylation of the receptor, suggesting a positive role for this isoform in EGF-mediated signals [189]. Increased DAG levels in DGK ϵ -deficient mice have a suppressive effect on transformation, as shown by reduced Ras-induced tumour formation in these mice, due to the activation of Rap GTPases [87].

FUTURE PERSPECTIVES

The DGK pathway modulates two lipids with important signalling properties that, in addition, are key intermediates in lipid metabolism. To accomplish their functions, an exquisite regulation governs the recruitment and activation of the different DGKs to various membrane compartments. DGK specificity and versatility relies on the presence of particular regulatory motifs, capable of binding ions or proteins or of imprinting a nuclear localization. These regions allow the different DGK isoforms to establish specific complexes and/or to be recruited to specific subcellular compartments. A major and exciting task in the near future will be to decipher the site of action as well as the signalling pathways regulated by each different DGK subtype.

Aberrant DGK function has been recently associated with pathological states, an expected consequence of the essential role of these enzymes in the regulation of multiple tissue and systemic functions. Recent discoveries place DGKs in the regulation of diseases that take a big toll on Western populations. Defining the contribution of DGK to the onset of these pathologies and identifying small-molecule modulators that target specific isoforms will enable great advances towards new treatments of malignant, heart, nervous and immune disease.

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