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Diacylglycerol kinase θ : Regulation and stability

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Abstract

Given the well-established roles of diacylglycerol (DAG) and phosphatidic acid (PtdOH) in a variety of signaling cascades, it is not surprising that there is an increasing interest in understanding their physiological roles and mechanisms that regulate their cellular levels. One class of enzymes capable of coordinately regulating the levels of these two lipids is the diacylglycerol kinases (DGKs). These enzymes catalyze the transfer of the γ -phosphate of ATP to the hydroxyl group of DAG, which generates PtdOH while reducing DAG. As these enzymes reciprocally modulate the relative levels of these two signaling lipids, it is essential to understand the regulation and roles of these enzymes in various tissues. One system where these enzymes play important roles is the nervous system. Of the ten mammalian DGKs, eight of them are readily detected in the mammalian central nervous system (CNS): DGK- α , DGK- β , DGK- γ , DGK- η , DGK- ζ , DGK- ι , DGK- ϵ , and DGK- θ . Despite the increasing interest in DGKs, little is known about their regulation. We have focused some attention on understanding the enzymology and regulation of one of these DGK isoforms, DGK- θ . We recently showed that DGK- θ is regulated by an accessory protein containing polybasic regions. We now report that this accessory protein is required for the previously reported broadening of the pH profile observed in cell lysates in response to phosphatidylserine (PtdSer). Our data further reveal DGK- θ is regulated by magnesium and zinc, and sensitive to the known DGK inhibitor R599022. These data outline new parameters involved in regulating DGK- θ .

Introduction

In addition to serving important roles in metabolism, diacylglycerol (DAG) and phosphatidic acid (PtdOH) are membrane lipids that serve important signaling roles (Almena and Merida, 2011; Cai et al., 2009; Carrasco and Merida, 2007; Wang et al., 2006). DAG binds to specific domains of various proteins, C1 domains, coupling to numerous and diverse signaling cascades that involve both GPCR and Tyrosine receptor kinase pathways (Brose et al., 2004). PtdOH has been implicated in numerous pathways that involve G proteins, vesicle regulation, and vesicular trafficking (see (Wang et al., 2006)). Diacylglycerol kinases are interfacial enzymes that are exquisitely poised to reciprocally modulate the relative levels of these signaling lipids. Currently, ten DGK isoforms have

been identified and many have known to regulate specific DAG effectors (see (Merida et al., 2008; Topham and Prescott, 1999)). Similarly, although discrete PtdOH-binding domains have not been identified, effectors of DGK-derived PtdOH are also known (Flores et al., 1996; Jones et al., 2000; Rainero et al., 2012). Events modulated by DGKs include T-cell activation and anergy (Zha et al., 2006), synaptic vesicle fusion (McMullan et al., 2006; Yang et al., 2010), trafficking (Hasegawa et al., 2008; Los et al., 2006), and gene expression (7, 8). Further, disruption of specific DGKs has been linked to various pathologies (Fuchs et al., 2011; Leach et al., 2007; Regier et al., 2005; Rodriguez de Turco et al., 2001). It is clear, therefore, that understanding of how these enzymes are regulated is essential to understanding the regulation of the involved signaling pathways.

One DGK isoform that has received relatively little attention is DGK- θ . This is the sole member of the Type V DGKs primarily distinguished by the fact it contains three C1 domains instead of two as observed in other isoforms. Further, although C1 domains are defined as phorbol ester/diacylglycerol binding regions (Cho, 2001; Cho and Stahelin, 2005; Geiger et al., 2003; Hall et al., 2005; Hurley, 2006), only two DGKs, DGK- β and DGK- γ , have been shown to bind phorbol esters (Shindo et al., 2003). For many years, our understanding of the factors regulating DGK- θ was limited to three components (a) inhibition by GTP-RhoA (Houssa et al., 1999), (b) translocation to cellular membranes (van Baal et al., 2005; Walker et al., 2001), and (c) interaction with acidic phospholipids – in particular PtdSer and PtdOH ((Tu-Sekine et al., 2007), and reviewed in (Tu-Sekine and Raben, 2011)). It's important to recognize, however, that these studies used intact cells or cellular lysates which compromises interpretations regarding enzyme regulation due to the complexity of the systems.

DGK- θ shows a striking predominate localization in the nervous system suggesting it plays a major role in this tissue. Consistent with this, evidence is accumulating that implicates mammalian DGKs in synaptic transmission (Biron et al., 2006; McMullan et al., 2006; Yang et al., 2010). The first evidence for a DGK- θ role in this process came from studies showing the DGK- θ homolog in *Caenorhabditis elegans* is a negative regulator of neurotransmitter release *in vivo* ((Miller et al., 1999; Nurrish et al., 1999) and see (Kano et al., 2002; Merida et al., 2008)). The potential role of DGK- θ in neurons underscores the need to understand the biochemistry and regulation of this enzyme.

In this report, we outline further studies of DGK- θ regulation and stability. In previous studies, we showed that this enzyme is regulated by PtdSer and PtdOH (Tu-Sekine et al., 2007). Further, PtdSer appeared to broaden the pH optimum of the enzyme (Tu-Sekine et al., 2006). Recent studies in our laboratory using purified DGK- θ indicate it is an auto-inhibited enzyme (*JBC, under revision*). Further, these studies demonstrate that full activity, and regulation by acidic phospholipids, requires an accessory protein that contains a polybasic region. In this report, we show that the broadening of the activity pH profile by PtdSer is dependent on the presence of an accessory protein.

Materials and methods

Materials

All lipids were purchased from Avanti. Silica gel 60 TLC plates were purchased from EM Science (Germany). Cytoscint scintillation-counting fluid was obtained from ICN (Costa Mesa CA). Histone H1 and cyclohexamide were purchased from EMD Biosciences (Santa Cruz, CA). Poly-L-lysine (low molecular weight) was purchased from Sigma. NP-40 was purchased from Pierce; NP-40 Alternative (NP-40alt) was purchased from EMD Biosciences. Dodecylmaltoside (DDM) was purchased from Invitrogen. All other chemicals were of reagent grade.

Cells and cell culture

Swiss 3T3 fibroblasts cells and neuroblastoma 2a (N2a) were grown and maintained as recommended by ATCC. Tissue culture media components were purchased from MediaTech, Inc. (Herndon VA). Plastic culture dishes were purchased from Falcon Labware.

DGK- θ assay, pH profile, and western blotting

These assays were performed essentially as previously described (Tu-Sekine et al., 2006, 2007). Vesicle composition was maintained at a 2:1 ratio of POPE:POPC, containing 9 mol % PtdSer, 8 mol % DAG (dioleoylglycerol, DOG). Assays were conducted at the following final concentrations unless otherwise noted: 7.5 mM lipid, 0.1 mM NaCl, 1 mM DTT, 50 mM Hepes, pH 7.0.

Free magnesium

All assays contained saturating Mg/ATP (1 mM). Free magnesium concentration was calculated based using Theo Schoenmakers' chelator algorithm (<http://www.stanford.edu/~cpatton/MgATP-TS.htm>). EDTA (0.4 mM) was added to one sample to chelate free magnesium and establish Mg-independent baseline activity.

Determination of DGK- θ half-life

Cyclohexamide (20 μ g/ml) was added to N2a and 3T3 cells 24 h after cells were seeded in 12W plates. Cells were collected at 0, 2, 4, 6, 24, 48, and 72 h and whole cell lysates were prepared for western blotting analysis. The relative amount of DGK- θ protein was determined with densitometry (Odyssey infrared systems, LiCor Biosciences) and normalized to GAPDH or α -tubulin. Curve fitting was done using Prism 5 software (GraphPad Software, San Diego CA).

Results and discussion

An accessory protein is required for PtdSer effects on the pH profile

PtdSer is known to modulate the activity of a number of DGKs and this is also true for DGK- θ (Tu-Sekine et al., 2007). For this enzyme, we demonstrated that PtdSer broadens the pH-dependent activity when the enzyme was assayed in cytosolic extracts (Tu-Sekine and Raben, 2010). Given our recent finding that the activity of DGK- θ is dependent on an

activating accessory protein containing a poly-basic region (PBR), we investigated the effect of activators on the pH profile. As shown in Fig. 1, the broadening of the pH profile of DGK- θ is also dependent on a PBR-containing activator.

Mg⁺² effects

Magnesium complexed ATP (MgATP) is an essential substrate for DGK- θ , as it is for all kinases. Recently, it has been suggested that magnesium may play another role in DGK catalysis. The crystal structure of a bacterial DGK, DGKB, indicates a binding site for Mg⁺² distinct from the MgATP binding site. Based on this structure, two aspartates are believed to participate in an Asp–water–Mg⁺² complex. In support of this, mutation of one of these two aspartates significantly diminishes catalysis in DGKB (Miller et al., 2008). Given these data, we examined the effect of free Mg⁺² on the activity of DGK- θ under conditions of saturating ATP (>0.5 mM) in the presence and absence of PtdSer (Fig. 2). We found that DGK- θ activity increased in the presence of excess magnesium. The K_M^{app} values for free Mg⁺² ranged from 0.047 ± 0.019 mM in the presence of PtdSer, to 0.310 ± 0.067 mM in the absence of PtdSer. The apparent decrease in the $K_{M(\text{free Mg}^{+2})}$ in the presence of PtdSer may be due to a charge-induced concentration of Mg²⁺ by the negatively-charged headgroup of PtdSer at the membrane interface, or may indicate that PtdSer increases the affinity of DGK- θ for Mg⁺². Our current data do not clearly distinguish between these possibilities. However, we have recently noted that decreasing the assay pH from 7.5 to 7.0 increases the $K_{M(\text{free Mg}^{+2})}$ which is consistent the hypothesis that PtdSer concentrates Mg²⁺ ions at the interface.

Inhibitors

We previously reported the effect of several commonly used inhibitors on the activity of DGK- θ (Tu-Sekine and Raben, 2010). We are now examining the effect of a number of inhibitors on the activity of purified DGK- θ . Importantly, we show that the DGK inhibitor, R59022, is an effective inhibitor of purified DGK- θ *in vitro* at concentrations 1 μ M under our conditions (Fig. 3B). This confirms several recent reports that implicate R59022, as well as R59949, in the inhibition of DGK- θ *in vivo* (Baldanzi et al., 2010). Further, ATP, ADP, sodium pyrophosphate, and β -glycerophosphate (BGP) were not effective inhibitors (Fig. 3), and phosphatase treatment only modestly decreased activity (Fig. 3C). The latter is particularly interesting given the report that this enzyme may be phosphorylated in a PKC-dependent manner (van Baal et al., 2005).

Given the presence of zinc-finger domains in DGKs (Merida et al., 2008), there has been an interest in the role of this metal in DGK activity. This is fueled by the knowledge that while the cysteine-rich (C1) domains of PKC bind DAG and phorbol esters in the presence of zinc, only DGK- β and DGK- γ are known to bind phorbol esters. These data led to the hypothesis that the cysteine-rich domains of DGKs have functions distinct from DAG binding. There is some evidence that zinc can play a non-catalytic role in DGK regulation. For example, the binding of zinc by the SAM domain of DGK- δ leads to the formation of an enzyme polymer, thereby inhibiting activity at membranes (Knight et al., 2010).

To determine whether zinc plays a role in DGK- θ , we examined the effect of zinc on purified DGK- θ activity. As shown in Fig. 4A, pre-incubation of DGK- θ with 100 μ M zinc inhibited the enzyme at pH 6-7.5. Zinc inhibits DGK- θ when the enzyme is pre-treated with zinc, or when zinc is added directly to the assay (Fig. 4B). Interestingly, zinc has been shown inhibit a store-operated calcium channel (SOCC) in brain with a K_i of 5 μ M (Kresse et al., 2005), which is within the range observed for DGK- θ inhibition. These data suggest that zinc may play a physiological role in regulating DGK- θ activity, and merits further investigation.

***In vivo* and *in vitro* stability**

In our study of DGK- θ , we have examined the stability of the enzyme both *in vivo* and *in vitro*. We find that the enzyme exhibits a mean half-life of approximately 5.1 ± 2.2 h in cultured cells (Fig. 5A). This is consistent with the reported mean half-life of cellular proteins (Eden et al., 2011). Interestingly, in addition to the expected 104 kD band identified by western blot using multiple DGK- θ antibodies, we have consistently observed bands near 70 kD and 35 kD in multiple cell lines, including IIC9 fibroblasts, HEK cells, N2a cells and primary neurons (Fig. 5C and data not shown). These fragments appear to represent DGK- θ , since the intensity of the bands diminished by western blotting following shRNA knock-down of DGK- θ . In addition, it appears that the 30 kD band represents an N-terminal portion of DGK- θ , while the 70 kD band contains the catalytic region, since the 30 kD band is detected by a polyclonal antibody raised against the intact protein (DGK- θ H-130), but not by a monoclonal antibody raised against a c-terminal region of human DGK- θ (a.a. 677-883; BD Biosciences 610931). Based on the size of the fragments the data suggest that DGK- θ is cleaved *in vivo*; however, we cannot exclude the possibility that these proteins are the product of transcript spliceforms, as has been reported for the 261 a.a. DGK- θ isoform CRA_b (Mural et al., 2002).

During purification and testing of DGK- θ , we identified a variety of compounds that stabilize the enzyme *in vitro*. Of note is the observation that purified DGK- θ is significantly more stable in the presence of an interface. In addition to polyethylene glycol (3500 MW), we found that DGK- θ activity appears to be improved when the enzyme is stored or briefly incubated prior to activity assay with detergents such as dodecylmaltoside (DDM) and pluronic F-127 (PF-127). Increasing the concentration of NP40 beyond a threshold resulted in loss of activity, but did not eliminate stimulation by a protein activator, suggesting enzyme denaturation has occurred (Fig. 6A-B). We have also explored the effects of incubation with phospholipid on DGK- θ activity, and find that the enzyme is stabilized during prolonged incubations in the presence of phospholipid vesicles, in a DAG-independent manner (Fig. 6C).

Conclusions

There is an increasing recognition of the roles of specific DGKs in a variety of signaling cascades (Shulga et al., 2011; Tu-Sekine and Raben, 2011). DGK- θ is a predominately neuronal enzyme and is one of the least understood of the ten known DGKs. Previous studies demonstrate that this enzyme is regulated by: GTP-RhoA-mediated inhibition

(Houssa et al., 1999), translocation to cellular membranes (van Baal et al., 2005; Walker et al., 2001), and activation by specific acidic phospholipids; PtdSer and PtdOH in particular ((Tu-Sekine et al., 2007), and see (Tu-Sekine and Raben, 2011)). The present report extends these studies and provides new insights regarding the regulation and stability of this enzyme. Clearly, however, much more is needed to understand the regulation and physiological role of this enzyme.

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Fig. 1.

Activators alter the pH profile of DGK-θ. (A) The presence of PtdSer (9 mol%) in vesicles broadens the pH profile for DGK-θ overexpressed in lysates (Tu-Sekine et al., 2006). (B) A similar profile broadening is observed in response to PtdSer using purified DGK-θ, when polylysine is also added to the assay, and (C) to a lesser extent when polylysine is added to vesicles that do not contain PtdSer. Experiments completed in triplicate, Error = SEM.

Fig. 2.

Effect of free magnesium on purified DGK-θ activity. (A) Representative profiles from 2 independent experiments in the presence (triangles) or absence (squares) of 9 mol% PtdSer show free magnesium dose-dependence on activity (pH 7.5). Calculated K_M^{app} values for free Mg in the presence (gray bar) and absence (white bar) of PtdSer (9 mol%). Error = SEM.

Fig. 3.

Modulation of DGK- θ activity *in vitro*. Purified DGK- θ was pretreated for 15 min with (A) ATP (1 mM), ADP (0.25 mM), NaPPI (0.5 mM) or BPG (1 mM) prior to assay. (B) DGK- θ was pretreated for 20 min with R59022 prior to an activity assay at pH 8.0 with R59022. (C) Purified DGK- θ was pretreated with 1 U CIP or CIP buffer only (control) for 10 min at 25 °C prior to an activity assay at pH 7.0. All preincubations contained 0.1% NP40. BPG = betaglycerolphosphate. Error = SD.

Fig. 4.

Purified DGK- θ is inhibited by zinc. (A) DGK- θ was incubated with 100 μ M zinc for 10 min. 100 μ M over a pH range prior to activity assay in the absence of additional zinc. Gray bars: control; black bars: zinc treated. (B) DGK- θ was incubated over a range of zinc concentrations prior to assay in the absence of zinc (black bars); or incubated with vehicle prior to assay in the presence of zinc (white bars). 0.1 μ M protamine present in all assays. Error = SD.

Fig. 5.

In vivo stability of DGK-θ. (A) Cyclohexamide treated 3T3 and N2a lysates were probed for endogenous DGK-θ by western blot over a 72 h time course (B) DGK-θ is evident as a 104 kD (full-length), 75 kD and 35 kD bands from western blots of primary cortical neurons.

Fig. 6.

DGK-θ activity is stabilized by the presence of an interface. Purified DGK-θ was incubated with detergents or PEG3500 for 10 min prior to assay. (A) Specific activity of treated enzyme (B) fold stimulation by histone H1 (0.1 μM) of treated enzyme. (C). DGK-θ was incubated in increasing concentrations of vesicles (9% PtdSer, 0% DAG (DOG), pH 7.5) for 1 h on ice, then for 30 min at room temperature prior to assay (pH 7.5, 0.1 μM Histone H1). Error bars = SD.