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Divergent and convergent signaling by the diacylglycerol second messenger pathway in mammals

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Diacylglycerol is an essential second messenger in mammalian cells. The most prominent intracellular targets of diacylglycerol and the functionally analogous phorbol esters belong to the protein kinase C family, but at least five alternative types of high affinity diacylglycerol/phorbol ester receptors are known: protein kinase D, diacylglycerol kinases α, β, and γ, RasGRPs, chimaerins, and Munc13s. These function independently of protein kinase C isozymes, and form a network of signaling pathways in the diacylglycerol second messenger system that regulates processes as diverse as gene transcription, lipid signaling, cytoskeletal dynamics, intracellular membrane trafficking, or neurotransmitter release.

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Abbreviations

DAG	diacylglycerol
ERK	extracellular signal regulated kinase
GPCR	G protein coupled receptor
IP_3	inositol 1,4,5-trisphosphate
JNK	Jun amino-terminal kinase
MEK	mitogen and extracellular signal regulated protein kinase kinase
Munc	mammalian uncoordinated
NF-κB	nuclear factor κB
PC	phosphatidylcholine
PE	phorbol ester
PH	pleckstrin homology
PKC	protein kinase C
PKD	protein kinase D
PLC	phospholipase C
PLD	phospholipase D
PtdIns	phosphatidylinositol 4,5-bisphosphate-specific
RasGRP	Ras guanyl nucleotide-releasing protein
RIN1	Ras interacting/interfering protein
SNARE	soluble N-ethyl maleimide sensitive factor attachment protein receptor

Introduction

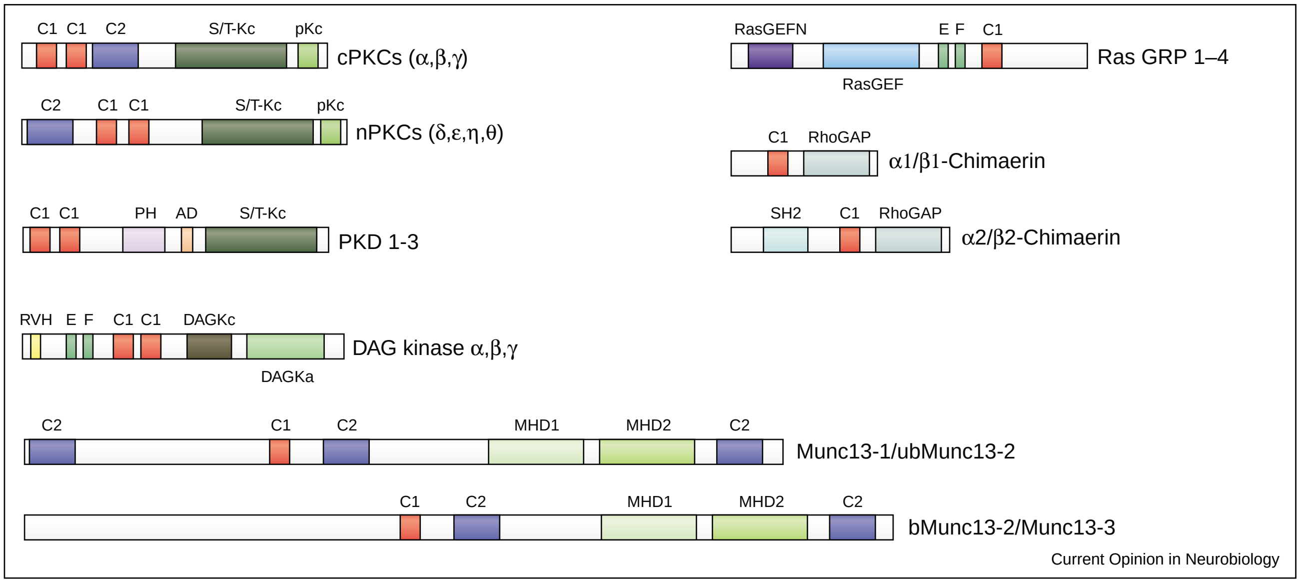
Diacylglycerols (DAGs) are glycerol derivatives that have two hydroxyl groups esterified by fatty acids. Under equilibrium conditions, biological membranes contain very few DAGs. Their production is stimulated upon activation of a multitude of cellular signaling cascades, and DAGs produced by these mechanisms act as important second messengers to modulate the function of target proteins.

Key enzymes in most of the signaling processes that generate DAG are the phosphatidylinositol 4,5-bisphosphate-specific phospholipase C isozymes PtdIns-PLCβ, PtdIns-PLCγ, PtdIns-PLCδ, and PtdIns-PLCε. They are activated by G-protein coupled receptors (GPCRs), receptor protein tyrosine kinases/non-receptor protein tyrosine kinases, Ca^{2+}, and Ras, respectively [1]. In addition, DAGs are produced from phosphatidylcholine (PC) by two subsequent reactions involving two mammalian PC-specific phospholipase D isozymes (PC-PLDs) and phosphatidic acid phosphohydrolase [2,3]. These are activated by a plethora of cellular signaling cascades involving cell surface receptors for signaling molecules, and intermediate activation of Arf and Rho type small GTPases as well as of protein kinase C (PKC) α and β [4–6].

Induction of PtdIns-PLC enzymatic activity causes the formation of DAG and inositol 1,4,5-trisphosphate (IP_3). IP_3, in turn, leads to the mobilization of Ca^{2+} from intracellular stores and DAG is able to bind to C_1 domains of a large number of proteins with diverse functions. The most prominent DAG targets belong to the PKC family of serine/threonine kinases. Binding of DAG, often in synergy with Ca^{2+}, leads to the membrane translocation and activation of certain PKCs [7–9]. PKCs are thought to regulate a multitude of neuronal processes, ranging from gene expression to proliferation, differentiation, apoptosis, adhesion, receptor and ion channel function, and transmitter release.

Modulation of cellular processes by DAG and by the functionally analogous phorbol esters (PEs), is usually attributed to activation of PKCs. Unlike all other second messenger pathways, in which paradigmatic targets are known but the fact that alternative targets are abundant and important is well appreciated, the DAG second messenger pathway is almost invariably discussed in the context of PKC function only. This is a dramatic misconception. Most eukaryotic cells, including neurons, contain five alternative non-PKC types of DAG/PE targets: protein kinase D (PKD), diacylglycerol kinases

Figure 1



A C_1 domain containing high affinity DAG/PE receptors. Abbreviations: C1, protein kinase C conserved region 1; C2, protein kinase C conserved region 2; DAGKa, diacylglycerol kinase accessory domain; DAGKc, diacylglycerol kinase catalytic domain; EF, EF hand; GAP, GTPase-activator protein; GEF, guanine nucleotide exchange factor; GRP, guanyl releasing protein; pKc, protein kinase C terminal domain; RasGEF, guanine nucleotide exchange factor for Ras-like small GTPases; RasGEFN, guanine nucleotide exchange factor for Ras-like GTPases (N-terminal motif); RhoGAP, GTPase-activator protein for Rho-like GTPases; RVH, recoverin homology domain; S/T-Kc, serine/threonine protein kinases-catalytic domain; SH2, Src homology 2 domain.

(DGK) β and γ, Ras guanyl nucleotide-releasing proteins (RasGRPs), chimaerins, and mammalian uncoordinated 13 (Munc13s) (Figure 1; [10–12]). Moreover, the pharmacological tools used to study PKC function are often not sufficiently specific to exclude the involvement of other DAG targets in cellular processes that are thought to be mediated by modulatory effects of DAG or PEs on PKCs [12].

Here, we discuss the function of known non-PKC DAG receptors and their roles in defined cellular signaling processes in which the effects of DAG and phorbol esters are not mediated by PKCs. We propose that the reader views the DAG second messenger system as a network of interconnected and partially overlapping signaling pathways in which PKC and non-PKC DAG/PE receptors have complementary functions but interact at multiple levels.

Protein kinase Cs

The domain responsible for high affinity binding of DAG and PE was first discovered in PKC isozymes and named the C_1 domain (Figure 1). In addition to having a kinase domain, which is present in all mammalian PKCs including the atypical isoforms (ζ, λ), the classical (α, β, γ) and novel PKCs (δ, ε,η, θ) also contain a C_2 domain. In the DAG sensitive classical and novel PKCs, the C_1 domain consists of two zinc finger like repeats, each of which can form a single ligand binding site for DAG or PE. The ligand bound C_1 domain mediates membrane targeting and concomitant activation of the corresponding PKC isozymes by reversion of autoinhibition. Full activation of classical PKCs requires additional binding of Ca^{2+} and acidic phospholipids such as phosphatidylserine, which is mediated by C_2 domains [9]. In addition, the spatial distribution and function of PKCs are regulated by isozyme specific binding proteins [13,14].

Thousands of publications cover the role of PKCs in the central nervous system and hundreds are added to the list every year — this forms an enormous dataset, according to which established and putative PKC substrates include transcription factors, cytoskeletal components, enzymes, ion channels, transporters, ionotropic and metabotropic receptors, trafficking proteins, and adhesion proteins. Essentially, most neuronal processes are thought to be regulated by PKCs in one way or another, from neural precursor cell proliferation and nerve cell differentiation to apoptosis. The majority of the corresponding studies involved pharmacological interference with PKC function. A problem in this context is that the most frequently used pharmacological tools for PKC activation and inhibition, such as PEs and many indolocarbazoles and bisindolylmaleimides, are not sufficiently specific to unequivocally define PKC mediated physiological effects in any experimental paradigm [10–12]. In particular, the separation of PKC mediated effects from those caused by other C_1 domain containing DAG/PE receptors is difficult because classical and novel PKCs as well as PKDs, DAG kinases α, β, and γ, RasGRPs, chimaerins, and Munc13s bind PEs and C_1 domain antagonists such as calphostin C with similar affinities [10–12].

Compared to drugs that target C_1 domains, antagonists of the ATP binding sites of PKCs are more specific, and some even show significant isozyme specificity [15,16]. The same is true for PKC isozyme specific inhibitor and activator peptides that block or induce the interaction with anchoring proteins [13,17], but the potential of peptide interference is largely ignored in the current literature. Given the problems associated with pharmacological studies on PKCs, alternative approaches using antisense oligonucleotides to block PKC expression [15,16] or overexpression of dominant active and dominant negative PKCs [18] have been employed frequently and contributed to our current knowledge of PKC function in neurons. However, neither of these approaches is unproblematic (e.g. because of excessive overexpression levels, peptide concentrations, or partial knock-down).

As one of the conceptually most stringent approaches, mouse genetic analyses of PKC function have yielded important insights into the function of individual PKC isozymes. Unfortunately, from the neuroscientific point of view, only very few of the corresponding mouse lines were studied in a neurobiological context. The most detailed information concerning PKC mutations in mice is currently available on phenotypic changes in immunological parameters and general cellular signaling (e.g. PKCs α, β, δ, θ, ζ, and λ) [19]. PKCγ, one of the most prominent PKC isozymes in the brain, is involved in learning and memory processes but is not the relevant DAG/PE receptor in the control of transmitter release [12,19,20]. Interestingly, mis-sense mutations in PKCγ in humans, which might not cause loss of function, are associated with spinocerebellar ataxia type 14 [21•,22•,23•]. PKCε is involved in the regulation of γ-aminobutyric acid A ($GABA_A$) receptor function and in the regulation of nociceptor function. Recent evidence indicates a role of PKCθ in Ca^{2+} signaling and transcriptional regulation [24], and of PKCλ in the regulation of insulin signaling [25].

A surprising feature of all PKC deletion mutant mice is the fact that they show only very mild phenotypic alterations, particularly with respect to nervous system function. This is in contrast to most data obtained with pharmacological approaches using PEs as PKC activators, and with overexpression or antisense knock-down approaches. This discrepancy between datasets is usually explained by assuming a functional redundancy among the different PKC isoforms, a view that requires detailed examination of mutants with deletions in multiple PKC genes. However, it is equally likely that several of the published roles of PKCs are in fact caused by other signaling proteins. Indeed, at least in the case of the numerous studies in which PEs were the main pharmacological tools, it is likely that some of the observed effects are not due to PKCs, but rather to alternative targets of the DAG signaling pathway.

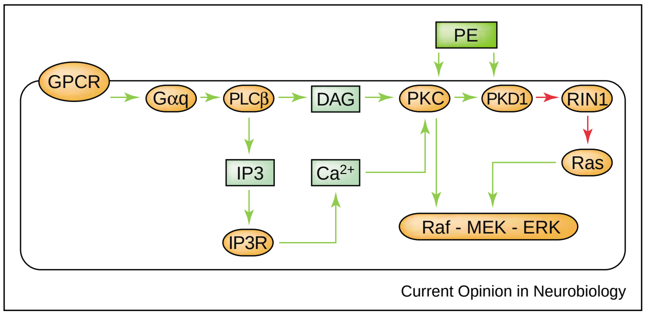
The analysis of an identified PKC target and its PKC phosphorylation site provides an escape from the dilemma in which studies on PKC function are often caught because of the redundancy problem of PKC mouse genetics on the one hand and the specificity problem of PKC pharmacology on the other hand. Once a PKC substrate and its PKC phosphorylation site are identified, phosphorylation-deficient and phosphomimetic mutants can be designed and analyzed, ideally in a wild type and in a deletion mutant background. This approach has recently resulted in the functional characterization of PKC mediated phosphorylation of the p65 subunit of nuclear factor κB (NF-κB; RelA) in NF-κB signaling, glutamate receptor GluR2 in synaptic transmission, and synaptosomal associated protein of 25 kDa (SNAP-25) and Munc18-1 in transmitter release [26•–29•].

Protein kinase Ds

The PKD family in mammals consists of PKD1 (also called PKCμ), PKD2, and PKD3 (also called PKCν). PKDs form a subfamily of AGC serine/threonine kinases that is distinct from other AGC superfamily members such as PKCs [30]. All PKDs contain two C_1 domains, a negatively charged central domain, a pleckstrin homology (PH) domain, and a serine/threonine protein kinase catalytic domain (Figure 1). PKD1 contains an additional amino-terminal apolar domain. Like PKCs, PKDs are important DAG/PE receptors whose function and subcellular localization are tightly controlled by DAG (and PEs).

Individual domains within the regulatory part of PKDs exert autoinhibitory effects on kinase activity that can be reverted by different activating signals. The major PKD activation mechanism involves phosphorylation by PKCα, PKCε, and/or PKCη at sites in the regulatory domain and activation loop of the kinase domain, which is triggered by receptor mediated PtdIns-PLCβ/PtdIns-PLCγ activation and DAG production [30]. This functional interaction between PKDs and PKCs is an important example of mechanistic coupling between two types of DAG/PE receptors (Figure 2). It can be triggered *in vivo* by GPCRs (through activation of PtdIns-PLCβ), growth factor receptors, B cell- and T cell-receptors (through activation of PtdIns-PLCγ), and other mechanisms [30]. Autoinhibition of the PH domain is reversed by direct binding of Gβγ subunits [31], by its direct tyrosine phosphorylation [32], or by phosphorylation of the activation loop [30]. In addition, PKDs are activated by binding of DAG and its PE analogues to the C_1 domain. Furthermore, caspase mediated cleavage can lead to PKD activation upon the induction of apoptosis by various drugs [30].

Figure 2



Potential crosstalk between PKCs and PKDs in signaling pathways to ERK. Certain PKCs, activated by GPCRs and DAG synthesis following PLCβ activation, can phosphorylate PKD1 and thus indirectly activate the Raf-MEK-ERK pathway through induction of RIN1 phosphorylation by PKD1. In addition, PKCs can directly phosphorylate Raf to activate the Raf-MEK-ERK pathway. PEs stimulate classical/ novel PKCs as well as PKDs. Abbreviations: IP3R, inositol 1,4,5-trisphosphate receptor.

The two C_1 zinc fingers of PKDs appear to have different functions. In PKD1, the first zinc finger acts as an autoinhibitory domain and is involved in DAG/PE-mediated and phosphorylation dependent activation, whereas the second zinc finger acts as a high affinity DAG/PE receptor essential for DAG/PE-dependent translocation (see below). Thus, in contrast to classical and novel PKCs, translocation and activation of PKDs are two functionally separate features [30]. The subcellular localization of PKDs is regulated by defined interactions with membrane lipids and proteins. In particular, the C_1 domains with their different lipid binding characteristics are of key importance in these processes. At rest, PKD1 is mainly present in the cytosol and to a lesser extent in the Golgi apparatus. Activation of GPCRs and activation of PtdIns-PLCβ by mitogenic agonists leads to a translocation of PKD1 from the cytosol to the plasma membrane, and this is most likely to occur through an interaction of the second C_1 zinc finger with DAG. Membrane association of PKD1 is followed by PKC-mediated phosphorylation of PKD1 and its accumulation in the nucleus, again a process that depends on the second C_1 domain. Export of PKD1 from the nucleus but not membrane association requires the PH domain. In addition to plasma membrane and nuclear translocation, PKD1 also binds to the trans-Golgi network in a manner that is dependent on local DAG synthesis and the first C_1 domain [30,33•,34•]. The two other PKDs show different subcellular distributions and responses to extracellular stimuli. One the one hand, PKD2 appears to lack the ability to perform activation-triggered nuclear translocation [35]. PKD3, on the other hand, is already present in the nucleus under resting conditions, and recruited to the plasma membrane and nucleus upon activation [36].

So far only very few established PKD targets are known. A prominent one is c-Jun, which is phosphorylated by PKD1 at sites that are distinct from those targeted by Jun amino-terminal kinase (JNK) and whose phosphorylation by PKD1 might lead to the downregulation of signaling through JNK in certain cell types. A second important PKD target is the Ras interactor RIN1 that dissociates from Ras upon PKD1-dependent phosphorylation, thereby activating the Ras/Raf- (mitogen and extracellular signal regulated protein kinase kinase-extracellular signal regulated kinase) MEK-ERK signaling pathway (see below) [30]. In addition, PKDs might phosphorylate myristoylated alanine-rich C kinase substrate and regulate permeability in lung microvascular endothelial cells [37].

Although the corresponding evidence is often still fragmentary and relies mainly on protein overexpression studies, PKDs are thought to be involved in the regulation of several cellular processes, including cell proliferation and survival, cell migration, immune response, and intracellular membrane trafficking. The role of PKDs in cell proliferation is probably attributable to their regulatory role in the ERK and JNK pathways. Activation of the ERK pathway is likely to occur by phosphorylation of the Ras interactor RIN1, which then dissociates from Ras and binds to 14-3-3 proteins, such that Ras can activate the Raf-MEK-ERK signaling pathway. Inhibition of the JNK pathway, which does not occur in all cell types, is thought to be caused by PKD-mediated phosphorylation of epidermal growth factor (EGF) receptors, thereby stopping further signaling to JNK, and by binding of PKDs to JNK, which inhibits JNK kinase activity and c-Jun phosphorylation [30]. However, alternative models envision a PKC-independent activation of JNK induced by bone morphogenic proteins and mediated by PKDs [38], or direct phosphorylation of c-Jun [39], possibly after recruitment of PKDs to the COP9 signalosome, a nuclear multiprotein complex that is essential for cell differentiation and development, where they might regulate c-Jun ubiquitination by direct phosphorylation [40]. PKD signaling in cell survival is triggered by oxidative stress, followed by Src activation, PtdIns-PLCγ-mediated DAG synthesis, PKC activation, and activation of PKDs through PKC-mediated phosphorylation in the activation loop [30]. Alternatively, or in addition, tyrosine phosphorylation of PKDs in the PH domain after activation of the Src–Abl signaling pathway could contribute to PKD activation. PKDs activated by these two survival pathways are thought to activate NF-κB through IKKβ (IκB kinase β) and IκB (inhibitor of κB)-degradation and thus lead to cell survival [41]. The regulation of cell shape and cell migration by PKDs appears to be relevant for cancer cell invasion. Here, PKDs were found to interact with cortactin and paxillin at membrane protrusions of invasive cancer cells where proteolysis of extracellular matrix occurs. The role of PKDs in immune responses is based on the fact that signaling through B-cell and T-cell receptors leads to the phosphorylation of PKDs through intermediate activation of tyrosine kinases, PtdIns-PLCγ, and PKCs [30]. Transgenesis experiments in mice showed that depending on its intracellular localization, PKD1 affects different aspects of T-cell proliferation and differentiation, including gene expression and somatic recombination [42].

A well characterized function of PKDs is their regulatory role in the Golgi apparatus where they are required for transport vesicle formation and transport of proteins from the Golgi apparatus to the plasma membrane [31,34•,43]. According to a current model, PKD1 is recruited to the Golgi apparatus by binding of its first C_1 domain to DAG in the Golgi membrane. In turn, PKD1 might then form a budding complex by recruiting effector proteins such as PtdIns-kinases that produce phosphorylated inositol phospholipids. The DAG that recruits PKD1 could also serve as a substrate for DAG kinases and be converted to phosphatidic acid. Phosphorylated inositol phospholipids (e.g. by recruitment of adaptor proteins) and phosphatidic acid (e.g. by inducing membrane curvature) could then contribute to membrane deformation, formation of short tubules, and finally vesicle fission [30,44]. Recent evidence shows that the regulatory effect of PKDs on membrane exit from the trans-Golgi network is important for basolateral sorting of membrane proteins [45•]. In addition, PKDs regulate cell motility by controlling anterograde membrane trafficking [46•].

Unfortunately, the function of PKDs in the central nervous system has not been examined yet. This lack of information is in part because overexpression studies are more difficult in nerve cells and pharmacological tools are not available. It is likely that this situation will change dramatically once suitable genetic tools such as deletion mutant mice are available.

Diacylglycerol kinases

DAG kinases phosphorylate DAG to form phosphatidic acid. DAG kinases have a major role in intracellular signaling in which they terminate the DAG signal generated by PLCs and form phosphatidic acid, which itself is a signaling molecule. As regulators of DAG levels and signaling, DAG kinases interfere with the function of all known high affinity DAG/PE receptors. In addition, some isozymes are also targets of DAG mediated regulation [47–49].

The family of mammalian DAG kinases comprises nine isozymes that form five subfamilies in mammals (I: α, β, γ; II: δ, η; III: ε; IV: ζ, ι; V: θ) (Figure 1). The different isozymes have a complex and partly overlapping expression pattern. Their function has mainly been examined in overexpression and pharmacological studies. All DAG kinases contain at least two C_1 domain zinc fingers (three in the type V DAG kinase θ) and a catalytic domain, which is interrupted by coiled coil domains in the type II isozymes δ and η. In addition, DAG kinases of the I, II, IV, and V type contain several different regulatory domains that are involved in lipid and protein interactions and that determine isozyme specific functions by regulating subcellular localization and sensitivity to different signaling pathways. Type I DAG kinases have an amino-terminal recoverin homology domain and two EF-hands, which function as Ca^{2+}-regulated autoinhibitory regions. Type II isozymes carry an amino-terminal PH domain, a carboxy-terminal sterile α motif, and four interspersed coiled coil domains. Type IV enzymes are characterized by the presence of carboxy-terminal ankyrin repeats and a central region with homology to myristoylated alanine-rich C kinase substrate, whereas the type V DAG kinase has an amino-terminal glycine/proline rich domain and a central PH-like Ras-associating region (Figure 1; [47–49]). Experiments with recombinant protein fragments showed that of the known DAG kinases only the β and γ isozymes have C_1 domains that function as high affinity DAG/PE receptors [50]. However, DAG kinase α appears to be recruited to membranes by DAG, indicating that its C_1 domain functions as a DAG receptor [47–49].

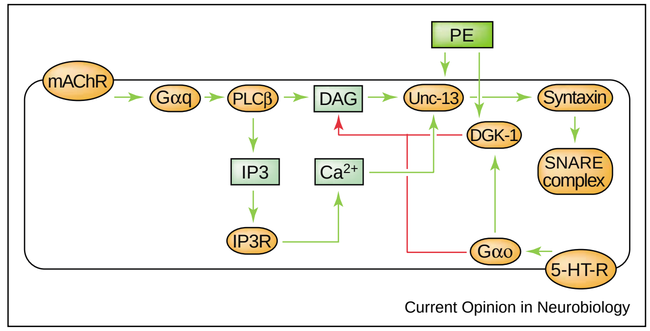
DAG kinases are thought to be active only in spatially restricted compartments following physiological DAG generation. One such subcellular compartment is the cytoskeleton where DAG kinases might regulate cytoskeletal dynamics by producing phosphatidic acid, which activates PtdIns-5 kinases. The resulting increases in PtdIns(4,5)P_2 would affect actin capping proteins and actin polymerization. In addition, DAG kinase dependent regulation of DAG and phosphatidic acid levels affect GTPase activating proteins for Rho family members (e.g. chimaerins, see below), and certain DAG kinases interact directly with Rho GTPases. In addition to a role in cytoplasmic signaling, DAG kinases are also constitutively localized in or recruited to nuclear compartments where they are thought to regulate a strictly compartmentalized DAG signaling pathway that controls cell proliferation [48].

Among the type I DAG/PE sensitive DAG kinases, the α isozyme is involved in T-cell receptor and hepatocyte growth factor receptor signaling and thereby regulates T-cell proliferation and endothelial cell function. In the case of T-cell receptor signaling, DAG kinase α functions as a signaling terminator. It is recruited to the membrane of T-cells by DAG, which originates from PLCγ activation, and released from membranes after DAG phosphorylation. In the case of hepatocyte growth factor receptor signaling, DAG kinase α is activated by Src [47–49]. Recent evidence indicates that DAG kinase α is not only regulated by Ca^{2+} but also by phosphoinositides generated by PtdIns-3-kinase activation [51]. A direct functional coupling between type I DAG kinases and DAG receptors of the RasGRP family was suggested on the basis of data obtained in T-lymphocytes, where DAG kinase α is able to adjust DAG levels and thus regulate Ras signaling by controlling the activity of the DAG/PE receptor RasGRP1 [52•]. DAG kinase γ, on the other hand, might participate in macrophage differentiation [53].

As mentioned above, the type II–V DAG kinases themselves are probably not directly regulated by DAG because their C_1 domains do not function as high affinity DAG/PE receptors. However, because of their role as DAG signaling terminators, they interfere with the function of other DAG/PE receptors. Moreover, some type II–V DAG kinases are regulated by PKCs. The type IV DAG kinase ζ is one of the best characterized DAG kinases. It is phosphorylated by PKCα, which leads to inhibition of DAG kinase activity and has profound effects on cell growth [54•,55•]. One pathway of DAG kinase ζ activation involves signaling thorough Src [56]. Similar to DAG kinase α, DAG kinase ζ is able to regulate Ras signaling by reducing DAG-dependent RasGRP activity [57]. This feature of DAG kinase ζ is likely to be responsible for its role in attenuating T-cell receptor signaling, which was demonstrated recently in T-cells lacking DAG kinase ζ [58•,59•]. The type II DAG kinase δ, which like DAG kinase ζ is also a PKC substrate [60], is thought to regulate membrane traffic from the endoplasmic reticulum to the Golgi apparatus, and DAG kinase θ, a type V isozyme, might be involved in complex lipid signaling processes because it is inhibited by RhoA, an activator of PLD [47–49].

The role of DAG kinases in neuronal function is only poorly understood. Genetic evidence in mammals indicates that the arachidonyl-DAG preferring type III DAG kinase ε participates in the regulation of synaptic transmission. Mice lacking this isozyme exhibit mild phenotypic changes characterized by altered lipid profiles due to changes in PLA_2-, PLC-, and PLD-pathways. By an as yet unknown mechanism, this results in reduced perforant path long-term potentiation and resistance to electroconvulsive shock [61]. Genetic evidence in *Caenorhabditis elegans* indicates that the nematode DAG kinase 1 participates in and influences DAG signaling induced by netrin/Unc-6 [62] and serotonin receptors [63,64], and thereby influences axonal branching and motor neuron function. In motor neuron function, the DAG/PE receptor Unc-13 is a likely DAG target whose function is attenuated by DAG kinase 1 action. This functional interaction between DAG kinase 1 and Unc-13 represents a fascinating type of crosstalk among DAG/PE receptors (Figure 3), but it is currently unclear whether it also occurs in mammalian neurons.

Figure 3



Potential crosstalk between diacylglycerol kinases and Unc-13 in SNARE complex regulation in *C. elegans* motor neurons. Muscarinic acetylcholine receptors activate PLCβ. The DAG generated by PLCβ activates Unc-13, which in turn activates Syntaxin. This results in more efficient SNARE complex formation and vesicle priming. Unc-13 may also be activated by Ca^{2+} binding to its C_2 domains, and by Ca^{2+}/calmodulin. Antagonistic serotonin receptors activate diacylglycerol kinase that reduces DAG levels and inhibits Unc-13. PEs stimulate DGK-1 as well as Unc-13. Abbreviations: 5-HT-R, serotonin receptor; IP3R, inositol 1,4,5-trisphosphate receptor; mAChR, muscarinic acetylcholine receptor.

Ras guanyl nucleotide-releasing proteins

The RasGRP family contains four isoforms, RasGRP1–4, that are characterized by an amino-terminal guanine nucleotide exchange factor domain for Ras-like GTPases (RasGEFN/RasGEF), two EF hand motifs, and a carboxy-terminal C_1 domain (Figure 1). A splice variant of RasGRP2 carries an amino-terminal palmitoylation/myristoylation site [65,66]. Through their RasGEFN/RasGEF domains, most RasGRPs promote GDP/GTP exchange and activation of Ras and related small GTPases [65,67–70], which leads to the stimulation of the Raf-MEK-ERK cascade and to the regulation of other signaling pathways. Calcium and DAG-regulated guanine nucleotide exchange factor I (CalDAG-GEFI), a splice variant of RasGRP2, activates Rap1a much more efficiently than Ras. Its GEF activity upon Rap1a is stimulated by PEs and Ca^{2+} and causes the inhibition of the Ras-induced Raf-MEK-ERK signaling pathway [10].

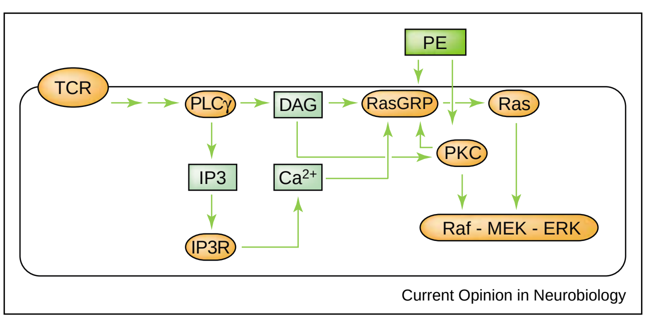
All RasGRPs contain C_1 domains whose structure is compatible with high affinity DAG/PE binding. Indeed, RasGRP1 and RasGRP3 were shown to be high affinity DAG/PE receptors [69,71], and RasGRP1, RasGRP3, and RasGRP4 translocate to membrane compartments in response to phorbol ester treatment [66,67,72].

Functionally, RasGRPs have been implicated in cell differentiation, cell proliferation, cell transformation [65,67,73], T-cell receptor signaling, T cell differentiation [74,75,76•,77], B-cell receptor signaling [78,79], integrin signaling [80], and neuronal differentiation of PC12 (pheochromocytoma cell line) cells [81]. All these functional roles are likely to be based on the Ras activating function of RasGRPs and the resulting activation of the Raf-MEK-ERK pathway, with the exception of

CalDAG-GEFI effects. Recent evidence indicates that RasGRPs, in particular RasGRP1, activate Ras specifically at Golgi membranes rather than the plasma membrane in a PLCγ dependent manner. RasGRP1 and RasGRP3 are present at and can be specifically targeted to Golgi membranes and other endomembranes where they are thought to activate Ras locally, leading to a sustained Ras activation that causes cell differentiation (e.g. in PC12 cells) rather than proliferation [82•,83,84,85•].

The best characterized signaling pathway that targets RasGRPs is triggered by T-cell receptors, propagated through the Rho-family GDP/GTP exchange factor Vav and PLCγ [52•,74,75,76•,77,86–88], and causes the activation of the Raf-MEK-ERK pathway independently of PKCs. In Ras signaling assays and assays of cell proliferation, mutant thymocytes that lack RasGRP1 are insensitive to PEs and T-cell receptor activation [74]. Moreover, loss-of-function mutations in RasGRP1 cause inadequate T-cell tolerance and autoimmunity [89•]. These genetic data demonstrate that DAG induced initiation of the Raf-MEK-ERK pathway — at least in thymocytes — is entirely dependent on RasGRP1 and unlikely to involve PKCs. The data obtained in RasGRP1 deletion mutant thymocytes provide the first direct and convincing evidence for a cellular DAG signaling pathway that is mediated by a non-PKC DAG/PE receptor rather than by PKCs as had been thought previously. It is likely that the same is true for other signaling pathways in other cell types, for example, in keratinocytes [90]. In any case, there is considerable crosstalk and overlap between PKC- and RasGRP-mediated pathways because PKCs can phosphorylate Raf and therefore also activate the Raf-MEK-ERK pathway, and because PKCs can directly phosphorylate RasGRPs (Figure 4; [91•]).

Figure 4



Potential crosstalk between PKCs and RasGRPs in signaling to ERK. DAG generated by T-cell receptors can recruit and stimulate RasGRPs and PKCs. The Ca^{2+} released from intracellular stores by IP3 can exert an additional activating effect on RasGRPs. Moreover, some RasGRPs are phosphorylated and activated by PKCs. RasGRPs directly activate Ras by promoting GDP/GTP exchange, and thereby activate the Raf-MEK-ERK signaling cascade. This effect converges with that of PKCs that can phosphorylate Raf directly. Some RasGRPs might specifically activate Ras on the Golgi membrane. PEs stimulate classical/novel PKCs as well as RasGRPs. Abbreviations: GRP, guanyl releasing protein; IP3R, inositol 1,4,5-trisphosphate receptor; TCR, T-cell receptor.

As is the case with several other non-PKC DAG/PE receptors, the role of RasGRPs in the brain is only poorly understood, although RasGRPs 1–3 are strongly expressed in the brain. Given their key functions in other organs, it is likely that RasGRPs contribute significantly to Ras signaling in the brain as well.

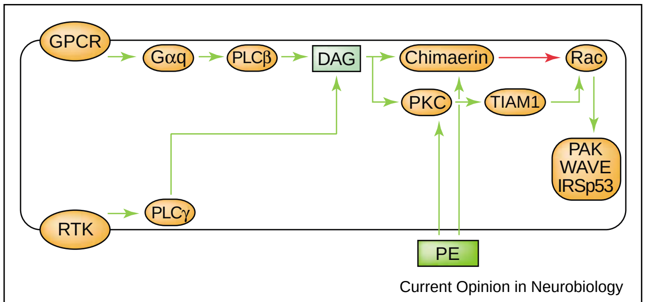
Chimaerins

Chimaerins, the first high affinity non-PKC DAG/PE receptors discovered, constitute a family of two isoforms (α and β), each of which is expressed as two splice variants (1 and 2). The type 1 chimaerins contain a C_1 domain and a Rac GTPase activating domain, whereas the type 2 isoforms have an additional amino-terminal SH2 domain (Figure 1; [10,11]). The C_1 domains of chimaerins are high affinity DAG/PE binding sites and chimaerins act as functional PE receptors when overexpressed in cells. β2-chimaerin, for example, translocates from a cytosolic compartment to the plasma and Golgi membranes after PE treatment. Translocation is dependent on an intact C_1 domain and an additional interaction with the Golgi transmembrane protein, Tmp21-I [92]. PEs do not affect the GTPase activating function of chimaerins, which indicates that the function of DAG binding is primarily to translocate chimaerins to membranes, thus spatially restricting its Rac GTPase activating effect [10,11,93].

The Rac GTPase activating function of most chimaerin variants is specific for Rac1 and not seen with Cdc42 or RhoA [93]. α1Chimaerin might also act upon Cdc42. By inactivating Rac, chimaerins necessarily interfere with all downstream effects of Rac (e.g. formation of lamellipodia and membrane ruffles, and loss of stress fibers). In this respect, their function competes with that of certain PKCs that can activate Rac by phosphorylation and activation of the GDP/GTP exchange factor Tiam 1 (Figure 5; [94]).

Chimaerins are implicated in diverse cellular processes such as cell adhesion, cytoskeletal dynamics, lamellipodia/filopodia formation, phagocytosis, and cell proliferation [10,11,95]. In the context of neuronal function, the modulatory effect of chimaerins on neuritogenesis in PC12 cells and neuroblastoma cells is particularly interesting. α2-Chimaerin induces neuritogenesis in these cells in an SH2 domain dependent manner [96]. It is likely that the Rac GTPase activating function is responsible for this effect, but direct evidence for a function of chimaerins in signaling to Rac *in vivo* is still lacking. Unfortunately, current information on the function of chimaerins is mostly derived from overexpression studies in non-neuronal cells. Nevertheless, the prominent effects of chimaerins on cytoskeletal dynamics and

Figure 5



Potential crosstalk between PKCs and chimaerins in the control of Rac activity. GPCRs and receptor tyrosine kinases (RTKs) induce DAG synthesis by the activation of PLCβ and PLCγ, respectively. DAG activates PKCs as well as chimaerins. The latter inactivate Rac, whereas PKCs act antagonistically by phosphorylating the RacGEF T lymphoma invasion/metastasis gene 1 (Tiam 1) and causing Rac activation, which results in activation of the p21 activated kinase (PAK) and Wiskott-Aldrich syndrome protein family verprolin homologous protein (WAVE) pathways and leads to lamellipodia formation and loss of stress fibers. PEs stimulate classical/novel PKCs as well as chimaerins. Abbreviations: IRSp53, insulin receptor tyrosine kinase substrate p53; PAK, p21-activated kinase; RTK, receptor tyrosine kinase; TIAM, T lymphoma invasion/metastasis gene 1; WAVE, Wiskott-Aldrich syndrome protein family verprolin homologous protein.

process formation in cell lines and the fact that chimaerins are strongly expressed in brain, where they could regulate Rac function, predict an important role of these proteins in axon and dendrite growth, navigation, and/or branching.

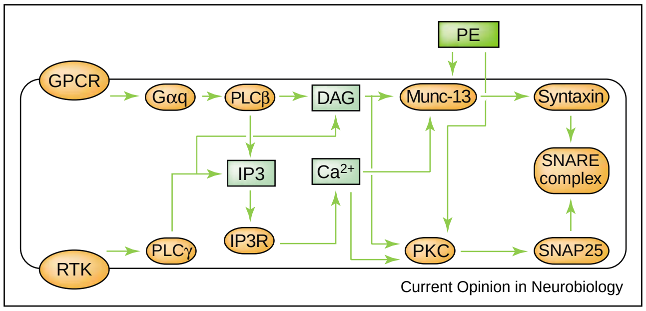
Munc13s

Munc13 proteins are homologs of *C. elegans* Unc-13. Mammals express three Munc13 isoforms that are specifically localized to presynaptic active zones, the transmitter secreting compartment of neurons (Munc13-1, -2, and -3; [12,97,98]). Munc13-2 is expressed as a brain specific (b) and ubiquitous (ub) splice variant. Munc13-1 and ubMunc13-2 contain an amino-terminal C_2 domain, a central C_1/C_2 tandem domain, two Munc13 homology domains, and a carboxy-terminal C_2 domain. bMunc13-2 and Munc13-3 each have unique amino-termini that lack a C_2 domain (Figure 1). Functional analyses in deletion mutant mice showed that Munc13s are essential for synaptic vesicle priming [98,99]. At the molecular level, Munc13s act by unfolding and activating the soluble N-ethyl maleimide sensitive factor attachment protein receptor (SNARE) protein Syntaxin and thereby promoting SNARE complex formation and secretory vesicle priming [97,100]. Munc13s are integrated into the presynaptic active zone network by interactions with other active zone components such as Rab3 interacting molecule (RIM), CAST/ERC (cytomatrix of the active zone/ proteins of the ELKS/Rab6-interacting protein 2/CAST family), and Bassoon, all of which might also exert regulatory effects on Munc13s [101,102•–105•].

All *bona fide* Munc13 isoforms bind PEs and DAG with high affinity and translocate to the plasma membrane in response to PE binding. Only the distantly related Munc13-4 variant and its homolog BAP3 have no C_1 domain and are therefore not targets of DAG signaling [106]. However, Munc13-4 has a typical Munc13 like priming function in cytolytic granule fusion. It is mutated in a form of familial hemophagocytic lymphohistiocytosis [107]. Mutation of the first histidine residue in the Munc13-1 C_1 domain (H567K) abolishes DAG/PE binding as well as PE dependent membrane translocation. Mutant mice expressing the DAG/PE binding deficient Munc13-1^{H567K} mutant instead of the wild type Munc13-1 from the endogenous *Munc13-1* locus die immediately after birth, demonstrating that an intact Munc13-1 C_1 domain is essential for survival. Hippocampal nerve cells from these mutants are almost completely insensitive to Pes, whereas wild type cells show robust increases in transmitter release in response to PE treatment. The residual PE sensitivity in homozygous Munc13-1^{H567K} cells is due to the presence of small amounts of Munc13-2 and eliminated completely in cells that express Munc13-1^{H567K} on a Munc13-2 deletion mutant background. Because expression and function of PKCs is unaffected in Munc13-1^{H567K} mutants and Munc13-1 is not a substrate of PE activated PKCs, these genetic data indicate that the PE induced augmentation of neurotransmitter release from hippocampal nerve cells is mediated exclusively by Munc13 proteins and not by PKCs [108•]. Thus, Munc13s rather than PKCs are the only functionally relevant PE and DAG sensitive presynaptic regulators of transmitter release. Similarly, insulin secretion from pancreatic β cells is increased by Munc13 action in a PE dependent manner [109].

The fact that homozygous Munc13-1^{H567K} mutant mice die immediately after birth demonstrates that Munc13-1 — unlike individual PKC isoforms — is an essential functional target of the DAG second messenger pathway in the brain. Detailed physiological analyses showed that the replacement of wild type Munc13-1 with a DAG binding deficient Munc13-1^{H567K} mutant leads to a reduction in the number of fusion competent vesicles, a stronger depression of synaptic transmitter release during high frequency action potential trains, and a reduction in the activity dependent refilling of the fusion competent vesicle pool [108•]. These data indicate that DAG dependent activation of Munc13-1 allows nerve cells to adjust their vesicle priming machinery to increases in activity levels. High frequency stimulation and concomitant Ca^{2+} influx or activation of presynaptic receptors appear to activate PtdIns-PLC isozymes (e.g. PtdIns-PLCδ and PtdIns-PLCβ) and thus lead to transient increases in synaptic levels of DAG, which in turn binds to the C_1 domain of Munc13-1 and boosts its priming activity [108•,110]. The fact that Munc13-1^{H567K} mutant mice die immediately after birth indicates that the C_1 domain

Figure 6



Potential crosstalk between PKCs and Munc13s in controlling SNARE complex function. GPCRs and RTKs trigger DAG synthesis and IP3/Ca^{2+} release through activation of PLCβ and PLCγ, respectively. DAG and Ca^{2+} activate both classical/novel PKCs and Munc13s. On the one hand, Munc13s activate Syntaxin, thereby causing more efficient SNARE complex formation and vesicle priming. PKC, on the other hand, phosphorylates SNAP-25 to increase SNARE complex formation and stability. PEs activate classical/novel PKCs as well as Munc13s. Abbreviations: IP3R, inositol 1,4,5-trisphosphate receptor; RTK, receptor tyrosine kinase.

dependent stimulation of Munc13-1 activity and the resulting adaptation to high activity levels is important for neurons involved in essential body functions (e.g. rhythmically active nerve cells in the respiratory system).

At the molecular level, DAG binding is likely to attach Munc13 proteins to the target plasma membrane of the active zone and thereby change their conformation and activation state — a process that would resemble the activation of PKCs by DAG [108•]. By positively regulating the SNARE component Syntaxin, the PE dependent action of Munc13s converges with that of PE dependent PKCs, which phosphorylate SNAP-25 and thereby also increase SNARE complex assembly and stability (Figure 6; [26•]).

Currently, the endogenous neurotransmitter systems and signal transduction pathways that target Munc13s in intact mammalian neuronal networks are unknown. Possible candidate mechanisms involve muscarinic and metabotropic serotonergic systems that appear to control the function of the *C. elegans* Munc13 homolog UNC-13 through $G_q\alpha$/PtdIns-PLCβ and $G_0\alpha$/DAG kinase 1, respectively (Figure 3; [63,64,111]). Indeed, presynaptic localization of the soluble Unc-13 MR splice variant appears to be regulated by DAG through the $G_0\alpha$/DAG kinase 1 pathway. Whether or not this crosstalk between DAG kinases and Munc13s also occurs in mammalian cells is unclear. In fact, an alternative mechanism is thought to be effective during high frequency activity in synaptic terminals of mammalian neurons. Here, the Ca^{2+} that accumulates during trains of action potentials might activate PtdIns-PLCδ that in turn generates DAG and activates Munc13s.

Conclusions

An overview of non-PKC DAG/PE receptors (Figure 1) and their functional interactions with each other and with PKCs (Figures 2–6) demonstrates that the DAG signaling system is a complex network of parallel, divergent, convergent, and overlapping pathways. These pathways are mediated by at least six different types of DAG receptors that are hard to distinguish from each other experimentally. As a consequence, the view that PKCs are the sole or even the most important targets of DAG/PE signaling is inadequate. This fact has to be taken into account when DAG signaling pathways are probed with pharmacological tools such as PEs. The main task in the future will be to dissect experimentally the DAG second messenger system and to identify the roles of individual DAG receptors and their interplay in specific cellular processes. This task can not be achieved with pharmacological tools but will rather require systematic genetic studies.

Update

Recent evidence from deletion mutant mice [112] indicates that PKC theta is involved in activity dependent synapse refinement and elimination at the neuromuscular junction.

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