Move over protein kinase C, you've got company: alternative cellular effectors of diacylglycerol and phorbol esters

Nils Brose[1,*] and Christian Rosenmund[2]

[1]Abteilung Molekulare Neurobiologie, Max-Planck-Institut für Experimentelle Medizin, D-37075 Göttingen, Germany
[2]Abteilung Membranbiophysik, Max-Planck-Institut für Biophysikalische Chemie, D-37077 Göttingen, Germany
*Author for correspondence (e-mail: brose@em.mpg.de)

Journal of Cell Science 115, 4399-4411 © 2002 The Company of Biologists Ltd
doi:10.1242/jcs.00122

Summary

Diacylglycerol is an essential second messenger in mammalian cells. The most prominent intracellular targets of diacylglycerol and of the functionally analogous phorbol esters belong to the protein kinase C (PKC) family. However, at least five alternative types of high-affinity diacylglycerol/phorbol-ester receptor are known: chimaerins, protein kinase D, RasGRPs, Munc13s and DAG kinase γ. Recent evidence indicates that these have functional roles in diacylglycerol second messenger signalling in vivo and that several cellular processes depend on these targets rather than protein kinase C isozymes. These findings contradict the still prevalent view according to which all diacylglycerol/phorbol-ester effects are caused by the activation of protein kinase C isozymes. RasGRP1 (in Ras/Raf/MEK/ERK signalling) and Munc13-1 (in neurotransmitter secretion) are examples of non-PKC diacylglycerol/phorbol-ester receptors that mediate diacylglycerol and phorbol-ester effects originally thought to be caused by PKC isozymes. In the future, pharmacological studies on PKC must be complemented with alternative experimental approaches to allow the separation of PKC-mediated effects from those caused by alternative targets of the diacylglycerol second messenger pathway. The examples of RasGRP1 and Munc13-1 show that detailed genetic analyses of C_1-domain-containing non-PKC diacylglycerol/phorbol-ester receptors in mammals are ideally suited to achieve this goal.

Key words: Protein kinase C, Phorbol ester, Munc13, Secretion, Synapse

Introduction

Diacylglycerols (DAGs) are glycerol derivatives in which two hydroxyl groups are substituted by fatty acids through ester bond formation. The physiologically relevant isomer is 1,2-diacyl-*sn*-glycerol of which mammalian cells contain many structurally distinct species that differ with respect to the type and degree of saturation of their fatty acid moieties. Ubiquitous DAGs are important intermediates in the synthesis and degradation of triglycerides, glycerophospholipids and glyceroglycolipids. De novo synthesis of DAG takes place in the endoplasmic reticulum.

Under equilibrium conditions, biological membranes contain very little DAGs. Their production is stimulated upon activation of a multitude of cellular signalling cascades, and DAGs produced by these mechanisms act as key second messengers to modulate the function of at least six different types of target protein, the most prominent of which belong to the protein kinase C (PKC) family (see below) [for reviews of DAG signalling see (Wakelam, 1998; Hodgkin et al., 1998; Goni and Alonso, 1999)].

Key enzymes in most of the DAG-generating signalling processes are the members of the phosphatidylinositol 4,5-bisphosphate-specific phospholipase C family (PI-PLCβ, PI-PLCγ, PI-PLCδ, PI-PLCε). Depending on the PI-PLC subtype, different cellular signalling molecules induce enzymatic activity via specific cell-surface receptors (see below). This enzymatic activity results in the hydrolysis of phosphatidylinositol 4,5-bisphosphate, which contains mainly polyunsaturated fatty acids, to inositol 1,4,5-trisphosphate and polyunsaturated DAGs. It appears that these polyunsaturated DAGs resulting from PI-PLC activity rather than the more saturated forms generated by alternative enzymatic pathways (see below) are most relevant as intracellular messengers targeting PKCs (for reviews, see Wakelam, 1998; Hodgkin et al., 1998). Whether non-PKC DAG targets show the same preference for polyunsaturated DAGs is not known but is likely, given the similarity in pharmacological characteristics of the respective binding sites (for reviews see Kazanietz et al., 2000; Kazanietz et al., 2002). Polyunsaturated DAG second messenger molecules are inactivated by the activity of diacylglycerol kinases (Wakelam, 1998; Hodgkin et al., 1998). Activation of PI-PLCβ isozymes is initiated by ligand binding to G-protein-coupled receptors. Relevant receptor systems include metabotropic receptors for classic neurotransmitters, monoamine receptors and receptors for numerous peptide signalling molecules. Apart from the G_q type α subunits coupled to these receptors, certain Gβγ subunits can also activate PI-PLCβ. PI-PLCγ activation involves phosphorylation by growth-factor-activated receptor protein tyrosine kinases or by non-receptor protein tyrosine kinases such as Lck/Fyn or c-Src. In the latter case, tyrosine kinase activation is mediated by G-protein-coupled receptors and

involves $G_s\alpha$ or $G_i\alpha$ subunits. PI-PLCγ isozymes can also be activated in a protein-tyrosine-kinase-independent manner, involving phospholipid-derived second messengers. Activation of PI-PLCδ isozymes is triggered by binding of Ca^{2+} to the EF-hand and C_2 domains of PI-PLCδ, followed by the association of the PH domain with phosphatidylinositol 4,5-bisphosphate. PLCε contains an RA domain that binds to Ras. Activation of Ras (e.g. following growth factor signalling) leads to translocation of PLC-ε to the plasma membrane and enzyme activation. In addition, PLCε is activated by $G\alpha_{12}$ (for a review, see Rhee, 2001).

Apart from the PI-PLC pathway, DAGs are produced from phosphatidylcholine, which predominantly contains saturated and mono-unsaturated fatty acids, by two subsequent reactions involving phosphatidylcholine-specific phospholipase D (PC-PLD) and phosphatidic acid phosphohydrolase (for reviews, see Wakelam, 1998; Hodgkin et al., 1998). As is the case for PI-PLCs, the two mammalian PC-PLDs (PC-PLD1 and PC-PLD2) are activated by a plethora of cellular signalling cascades, often involving cell-surface receptors for signalling molecules (for reviews, see Exton, 1998; Liscovitch et al., 2000; Cockcroft, 2001). However, the saturated/mono-unsaturated phosphatidic acid intermediates generated by PC-PLD activity appear to be mainly responsible for the cellular signalling events that are triggered by PC-PLD activation, whereas the saturated/mono-unsaturated DAGs resulting from the subsequent phosphohydrolase reaction may be irrelevant for signalling, at least as far as activation of PKCs is concerned. Thus, the activity of specific phosphatidic acid phosphohydrolases may lead to signal termination by inactivating saturated/mono-unsaturated phosphatidic acid (Wakelam, 1998; Hodgkin et al., 1998). In general, PC-PLD activation by cell-surface receptors is indirect and often mediated by G-protein-coupled receptors and G-protein activation. Depending on the cellular process, PC-PLD stimulation involves intermediate activation of Arf- and Rho-type small GTPases as well as of PKCs α and β. In addition, lipid-derived signalling molecules such as phosphatidylinositol 4,5-bisphosphate or oleate, which are the products of regulated kinase or lipase activities, stimulate PC-PLD (Exton, 1998; Liscovitch et al., 2000; Cockcroft, 2001).

Two additional cellular pathways of DAG production, both utilizing phosphatidylcholine, involve phosphatidylcholine-specific PLC (PC-PLC) and phosphatidylcholine-ceramide cholinephosphotransferase (for a review, see Wakelam, 1998). In both cases, mostly saturated/mono-unsaturated DAGs are produced, the signalling role of which is questionable (see above). Moreover, direct evidence for an involvement of DAGs generated by these pathways in mammalian cellular signalling events is sparse, particularly where signalling cascades that are triggered by the activation of cell surface receptors are concerned.

Considering the various possible sources of DAGs in mammalian cells, it is evident that polyunsaturated DAGs resulting from PI-PLC activity are the most relevant DAG second messengers. Irrespective of the PI-PLC isozyme involved, induction of enzymatic activity causes the formation of DAG and inositol 1,4,5-trisphosphate. Inositol 1,4,5-trisphosphate, in turn, leads to the mobilisation of Ca^{2+} from intracellular stores; DAG is able to bind to C_1 domains of a large number of proteins with diverse function. As mentioned above, 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 membrane translocation and activation of PKC isozymes (Newton, 1995; Newton, 1997; Newton, 2001). After activation, PKCs are thought to regulate a multitude of intracellular processes, ranging from cell proliferation to neurotransmitter and hormone secretion. Modulation of cellular processes by DAG and by the functionally analogous phorbol esters (natural diterpene

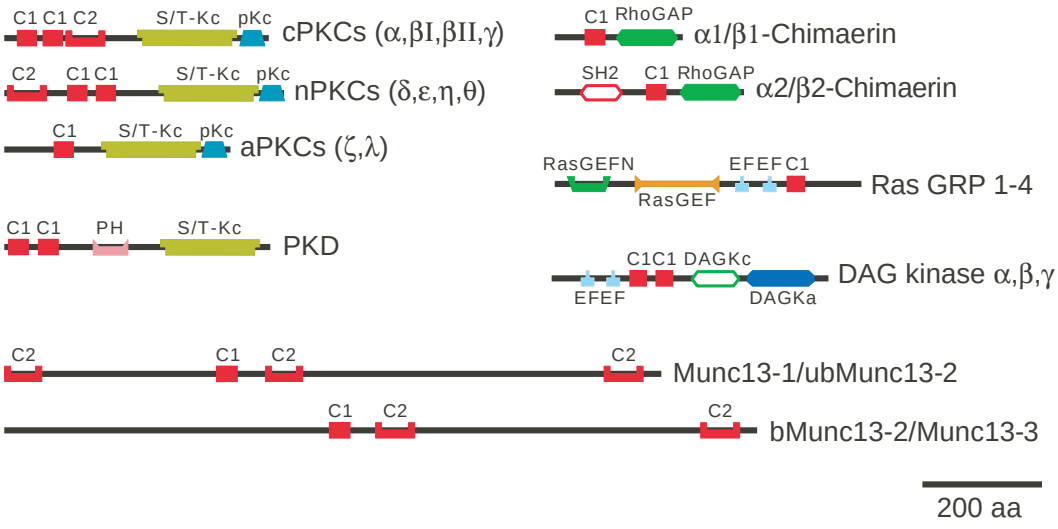

Fig. 1. C_1-domain proteins that bind DAG and phorbol esters with high affinity. C1, protein kinase C conserved region 1; C2, protein kinase C conserved region 2 (CalB); DAG, diacylglycerol; DAGKa, diacylglycerol kinase accessory domain; DAGKc, diacylglycerol kinase catalytic domain; EF, EF hand; GAP, GTPase-activator protein; GEF, guanine nucleotide exchange factor; GRP, guanyl-releasing protein; PH, pleckstrin-homology domain; pKc, protein kinase C terminal domain; PKC, protein kinase C; PKD, protein kinase D; 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; S/T-Kc, serine/threonine protein kinases, catalytic domain; SH2, Src homology 2 domain.

secondary metabolites of *Euphorbiaceae* and *Thymelaceae*, see below) has often been attributed exclusively to activation of PKCs. This is surprising because most eukaryotic cells contain five alternative types of DAG targets [chimaerins, protein kinase D1 (PKD1), RasGRPs, Munc13s and DAG kinase γ (Fig. 1) (for reviews see Kazanietz, 2000; Kazanietz, 2002; Kazanietz et al., 2000)], and the pharmacological tools that are frequently used to study PKC function are 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 phorbol esters on PKCs (Betz et al., 1998; Kazanietz, 2000; Kazanietz et al., 2000; Way et al., 2000; Rhee et al., 2002). Indeed, a number of observations indicate that the effects of DAG and phorbol esters are not mediated by PKCs but rather involve three alternative DAG targets in at least three key cellular processes: (1) DAG- and phorbol-ester-mediated subcellular translocation of PKD1 is essential for protein transport from the trans-Golgi network to the cell surface (Matthews et al., 1999a; Maeda et al., 2001; Rey et al., 2001; Baron and Malhotra, 2002; Van Lint et al., 2002); (2) activation of the Ras/Raf/MEK/ERK pathway in T lymphocytes is triggered by G-protein-coupled receptors and tyrosine-kinase-coupled receptors and is dependent on DAG- (or phorbol-ester-) induced activation of RasGRP rather than PKCs (Dower et al., 2000; Jones et al., 2002); (3) stimulatory effects of DAG and phorbol esters on neurotransmitter secretion from nerve cells are mediated by DAG/phorbol-ester receptors of the Unc-13/Munc13 family and not, as previously believed, by PKC isozymes (Betz et al., 1998; Lackner et al., 1999; Miller et al., 1999; Nurrish et al., 1999; Rhee et al., 2002).

As the relevance of non-PKC DAG receptors in the DAG second messenger pathway is appreciated only by a rather small circle of experts in DAG signalling but otherwise ignored, here we critique the widely accepted model according to which all DAG/phorbol-ester effects are caused by the activation of PKC isozymes, a view that is presented in most current textbooks and forms the (at least implicit) conceptual basis of almost all past and current pharmacological studies concerned with the determination of the role of PKC isozymes in the control of cellular function. We briefly summarise established and postulated functional roles of PKCs and discuss the characteristics of pharmacological tools that are routinely used in most studies of PKC function in vivo. Subsequently, we describe major caveats of pharmacological analyses of PKC function and discuss alternative and more powerful experimental approaches such as the use of dominant-interfering PKC variants, antisense knockdown of PKC expression or PKC gene deletion in mice, which have yielded important insights into PKC function but are not part of the methodological repertoire in most studies of PKC function. We conclude with a discussion of the functional importance of four non-PKC targets of the DAG second messenger pathway, chimaerins, PKD1, RasGRPs and Munc13s – with a focus on the latter.

PKCs as paradigmatic C_1-domain-containing DAG receptors

The protein sequence responsible for high-affinity binding of DAG and phorbol esters was initially discovered in PKC isozymes and designated the C_1 domain. Depending on the PKC type, the C_1 domain consists of one (aPKCs) or two (cPKCs and nPKCs) zinc-finger-like repeats that have a conserved pattern of cysteine and histidine residues and form a coordination site for two Zn^{2+} ions (H-X_{12}-C-X_2-C-$X_{13/14}$-C-X_2-C-X_4-H-X_2-C-X_7-C). Each individual zinc finger motif can form a single ligand-binding site for DAG or phorbol ester. Only the single C_1 domain motifs of aPKCs do not bind DAG, and their function remains elusive (Mellor and Parker, 1998; Newton, 2001). DAG or phorbol-ester binding to the C_1 domain does not significantly change its conformation but rather creates a contiguous hydrophobic surface on the side of the C_1 domain that carries the ligand-binding pocket by capping hydrophilic residues (Zhang et al., 1995) (for reviews, see Hurley and Meyer, 2001; Newton, 2001). The hydrophobic surface of ligand-bound C_1 domains then mediates membrane targeting and concomitant activation of the corresponding enzymes. Apart from DAG binding to C_1 domains, full activation of cPKCs requires Ca^{2+} and acidic phospholipids such as phosphatidylserine (Newton, 2001). Ca^{2+}-induced activation of cPKCs is mediated by the C_2 domain. This region forms a compact β sandwich fold that binds Ca^{2+} in a cup-shaped depression via five conserved aspartic acid residues. Ca^{2+} binding is thought to trigger a conformational change that opens a cleft to allow binding of an acidic phospholipid headgroup of a membrane phospholipid, thus mediating membrane targeting and activation of the corresponding PKC isozymes (Mellor and Parker, 1998; Newton, 2001). nPKC C_2 domains do not bind Ca^{2+} but mediate essential protein-protein interactions (e.g. with substrates such as GAP-43) (Mellor and Parker, 1998; Newton, 2001). In addition to C_1 domains and C_2 domains, the basic pseudosubstrate domains of cPKCs and nPKCs contribute to membrane interactions during kinase activation (Newton, 1997).

In all cPKCs and nPKCs, kinase activation is closely coupled to ligand binding by C_1 and C_2 domains and the resulting membrane translocation, which in turn reverses autoinhibition caused by a pseudosubstrate site. Essentially, the C_1 and C_2 domains of cPKCs and the C_1 domains of nPKCs can function as independent membrane-targeting modules such that ligand binding by each individual domain type leads to significant membrane association (Newton, 2001). Indeed, phorbol ester or DAG binding to the C_1 domains of nPKCs (and to a lesser degree also of cPKCs) is sufficient for translocation and activation. On the other hand, Ca^{2+}-dependent phospholipid binding by the C_2 domains of cPKCs acts synergistically with DAG binding to the respective C_1 domain. Such synergistic activation of cPKC C_1 and C_2 domains leads to the translocation and tight membrane association of the enzyme, which then causes a conformational change that reverts autoinhibition (Newton, 2001; Hurley and Meyer, 2001).

For proper cellular function of PKCs, their correct spatial distribution is essential. Such spatially specific targeting of PKCs is unlikely to be brought about by C_1- and C_2-domain-mediated membrane interactions alone. Rather, specificity of PKC membrane targeting is thought to be achieved by isozyme-specific binding proteins that are essential for the formation of PKC-containing subcellular signalling complexes at the appropriate subcellular location, thus spatially restricting PKC signalling and allowing integration of PKC-mediated

signalling with other intracellular signalling pathways. PKC-binding proteins regulate targeting of PKC to upstream activators (e.g. InaD, Syndecan-4), to substrates (e.g. STICKs, RACKs) or to cytoskeletal and vesicular proteins (e.g. actin) (for reviews, see Csukai and Mochly-Rosen, 1999; Jaken and Parker, 2000).

PKCs are thought to play essential roles in multiple cellular signal transduction pathways of eukaryotic organisms. Genetic studies in yeast have demonstrated that the dynamic regulation of the cell wall is the major cellular site of PKC action in this simple organism. Deletion of PKC in *Saccharomyces cerevisiae* leads to arrest of protein synthesis prior to mitosis but after DNA synthesis. The underlying signalling pathway is triggered by Hcs77p and involves the activation of PKC by Rho1. PKC activation, in turn, initiates a phosphorylation cascade via a MAP kinase module that leads to the activation of several transcription factors and transactivation of, among others, heat shock and cell wall genes (Mellor and Parker, 1998).

Genetic studies on PKC function in mammals are more difficult to interpret owing to the presence of multiple genes and possible functional redundancy. In fact, all known PKC-deletion mutants show rather mild phenotypic changes. Nevertheless, they have yielded important insights into the function of individual PKC isozymes. (1) PKCγ, one of the most prominent PKC isozymes in brain, has been shown to be important for brain functions involved in learning and memory (Abelovic et al., 1993). Interestingly, the typical stimulatory effects of phorbol esters on transmitter release are still detectable in PKCγ-deficient nerve cells, indicating that alternative DAG/phorbol-ester receptors are involved in this phenomenon (Goda et al., 1996). (2) Lack of PKCβ leads to immunodeficiency [impaired humoral response and cellular B cell response (Leitges et al., 1996)]. PKCβ appears to be critically involved in B-cell-receptor-mediated survival signalling to NF-κB (Su et al., 2002). Interestingly, B-cell-receptor-mediated signalling in PKCβ-deficient B cells can still be bypassed by phorbol esters, indicating the involvement of alternative DAG/phorbol-ester receptors in this pathway (Leitges et al., 1996). (3) PKCε has been shown to be involved in the regulation of $GABA_A$ receptor function (Hodge et al., 1999) and in the regulation of nociceptor function (Khasar et al., 1999). (4) PKCθ appears to be involved in a unique signalling pathway linking T cell antigen receptor signalling to NF-κB activation in mature T lymphocytes (Sun et al., 2000). (5) PKCδ-deficient smooth muscle cells exhibit increased apoptotic resistance (Leitges et al., 2001a). In addition, loss of PKCδ leads to increased antigen-induced mast cell degranulation (Leitges et al., 2002) and to the prevention of B cell tolerance owing to maturation and differentiation of self-reactive B cells (Mecklenbrauker et al., 2002). (6) PKCζ is important for the regulation of NF-κB transcriptional activity. As a consequence, lack of PKCζ leads to impaired B cell receptor signalling, inhibition of cell proliferation and survival and defects in the activation of ERK and the transcription of NF-κB-dependent genes (Leitges et al., 2001b; Martin et al., 2002).

Numerous studies using alternative approaches indicate the involvement of different PKCs in the modulation of ion channel conductance, transmitter receptor function, smooth muscle contraction, cell migration, cell proliferation and differentiation, apoptosis, lipogenesis, glycogenolysis, as well as transmitter/hormone exocytosis and protein secretion [for examples from the large number of reviews on PKC function in the literature, see (Kanashiro and Khalil, 1998; Dempsey et al., 2000; Barry and Kazanietz, 2001; Ventura and Maioli, 2001)]. Apart from insights into PKC function that have been obtained in pharmacological studies employing small molecule activators and inhibitors of PKCs (which have particular advantages and disadvantages as discussed below), many of the current models of PKC function originate from studies in which the role of individual PKC isozymes was characterised using more informative methodological approaches. These include the following: (1) overexpression of wild-type and dominant interfering PKC mutants [e.g. PKCζ as a regulator of RelA transcriptional activity (Anrather et al., 1999); PKCα and PKCδ as regulators of glucose transport (Tsuru et al., 2002); PKCα and PKCθ as regulators of calcineurin-induced transactivation (Ishaq et al., 2002); (for a review, see Dempsey et al., 2000)]; (2) interference with PKC expression using ribozymes [e.g. PKCα as a regulator of glioma cell growth (Sioud and Sorensen, 1998)]; (3) interference with expression using antisense oligonucleotides [for reviews of the literature with an emphasis on therapeutically relevant approaches see (Tamm et al., 2001; Goekjian and Jirousek, 2001; Swannie and Kaye, 2002)]; and (4) interference with PKC function using peptides that induce or block PKC interactions with targeting proteins (for reviews, see Csukai and Mochly-Rosen, 1999; Jaken and Parker, 2000).

All the above approaches are conceptually and experimentally more stringent than the classic pharmacological studies (see below), although they have their individual caveats. Overexpression often results in levels of wild-type or dominant-negative PKC variants that exceed endogenous levels by an order of magnitude or more. As a result, overexpressed wild-type PKC isozymes may participate in signalling processes that they are usually not involved in, and mutant variants (e.g. kinase-deficient mutants) may interfere in a dominant-negative manner with signalling to other targets of signalling pathways (e.g. the DAG second messenger pathway). These problems can be accounted for by complementing data obtained in overexpression studies with data obtained using deletion mutations of the corresponding PKC isoform under investigation. Ribozymes and particularly antisense oligonucleotides often yield only partial knockdown of expression levels. Peptides, by contrast, are often used at rather high concentrations such that non-specific effects must be excluded. Irrespective of the distinct advantages of these experimental approaches, the majority of studies of PKC function in different cell biological processes are not characterised by a comparable conceptual and experimental stringency. In most of these cases, which are typically concerned with the problem of whether PKCs in general are involved in a given cellular process, commercially available pharmacological tools are used to activate or inhibit PKCs. The main caveat with these pharmacological studies is that neither the almost exclusively used phorbol-ester-derived PKC activators nor many of the commonly used PKC inhibitors are specific for PKCs (see below).

Pharmacological tools to interfere with PKC function

DAG is one of the most important second messengers involved in PKC activation, and certain DAG-related cyclic lactones are

potent PKC activators. However, the most commonly used pharmacological tools for PKC activation belong to the phorbol-ester family of tumour promoters. Phorbol esters are secondary metabolites of *Euphorbiaceae* and *Thymeleaceae* and mimic the action of DAG at C_1 domains. In common with DAG, phorbol esters bind to the C_1 domain of PKCs and induce membrane translocation and activation of the enzyme (for reviews, see Hurley and Meyer, 2001; Newton, 2001; Barry and Kazanietz, 2001). In comparison with phorbol esters, efficacy and specificity of alternative PKC activators such as thymeleatoxin, sapintoxins A and D, 12-deoxyphorbol esters, mezerein, indolactam V, resiniferatoxin, tinyatoxin, thapsigargin or bistratene A are much lower (Way et al., 2000). Unfortunately, none of the commonly used phorbol esters is specific for PKCs. In fact, several alternative C_1-domain-containing proteins, including the chimaerin (Ahmed et al., 1990; Ahmed et al., 1993; Areces et al., 1994; Caloca et al., 1997; Caloca et al., 2001), Munc13 (Betz et al., 1998) and RasGRP (Ebinu et al., 1998; Lorenzo et al., 2000) protein families, as well as PKD1 (Valverde et al., 1994) and DAG kinase γ (Shindo et al., 2001), bind phorbol esters with PKC-like affinity (see below) (for reviews see Ron and Kazanietz, 1999; Kazanietz, 2000; Kazanietz, 2002; Kazanietz et al., 2000; Barry and Kazanietz, 2001). Thus, studies of PKC function that rely on the use of phorbol esters as an investigative tool have to be interpreted with caution. This is particularly pertinent for cellular processes that are also regulated by alternative DAG/phorbol-ester receptors (e.g. the regulation of intracellular vesicle transport by PKD1, the regulation of transcription by RasGRPs or the regulation of neurotransmitter release by Munc13 isoforms).

As is the case for C_1-domain-directed PKC activators, C_1-domain-directed PKC inhibitors are non-specific pharmacological tools that bind with comparable affinity to other C_1 domain proteins. Such non-specific inhibitors include one of the PKC inhibitors used most widely in the past, calphostin C (Betz et al., 1998; for a review, see Barry and Kazanietz, 2001). For the functional separation of PKC-specific effects from those mediated by alternative DAG/phorbol-ester receptors, some of the most useful pharmacological tools are ATP-binding site inhibitors. Although many of these (e.g. the indolocarbazole staurosporin, some balanol analogs, phenylaminopyrimidines, and rottlerin) inhibit protein kinases non-specifically, certain indolocarbazoles (e.g. Midostaurin/CGP41251, Gö6976, Gö7612, Gö7874, UCN-01) and bisindolylmaleimides (Gö6850, Gö6983, LY-333531, LY-379196, LY-317615) are rather PKC specific, some even show a preference for certain isozymes (Kanashiro and Khalil, 1998; Barry and Kazanietz, 2001; Way et al., 2000; Goekjian and Jirousek, 2001; Swannie and Kaye, 2002).

The main problem with some of the most specific bisindolylmaleimide-derived PKC inhibitors is their partial toxicity in certain situations. Gö6859, for example, causes a dramatic nonspecific rundown of synaptic transmission in primary hippocampal nerve cells without affecting phorbol-ester effects in this system (Fig. 2) (Rhee et al., 2002). Given that most PKC inhibitors are usually applied according to a preincubation paradigm (i.e. for minutes) and at rather high concentrations, even mild nonspecific or toxic effects of such drugs can have profound consequences. Nevertheless,

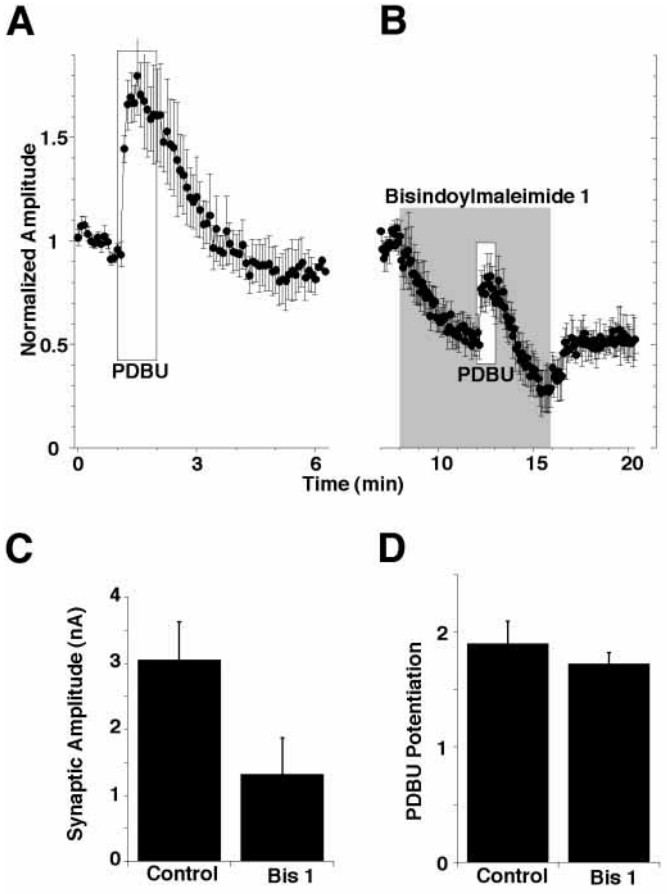

Fig. 2. Nonspecific effects of a bisindolylmaleimide-derived PKC inhibitor in hippocampal neurons. (A) Time course of phorbol-ester effects on evoked EPSCs in wildtype neurons (*n*=6). Application of PDBU (1 μM) is indicated by the white box. (B) Preincubation with 3 μM bisindoylmaleimide I (Gö6857, grey box) led to a partially irreversible rundown of evoked EPSC amplitudes but did not block the potentiation induced by application of 1 μM PDBU (white box). (C) Average evoked EPSC amplitudes in untreated neurons before and after bisindoylmaleimide I (3 μM) pretreatment (*n*=6). (D) Average phorbol-ester-dependent potentiation of evoked EPSC amplitudes (30 seconds following onset of application of 1 μM PDBU, *n*=6) in untreated and bisindoylmaleimide I (3 μM) pretreated neurons from experiments shown in (B). Error bars indicate s.e.m.

the indolecarbazol CGP41251/midostaurin and the bisindolylmaleimide LY-333531 have advanced to late-stage clinical development for the treatment of cancer and other indications, demonstrating that these drugs act quite specifically under carefully controlled conditions (for reviews, see Goekjian and Jirousek, 2001; Swannie and Kaye, 2002).

Apart from small molecule inhibitors of PKC, a recently emerged alternative pharmacological approach to perturb PKC activity involves peptides that interfere with the membrane translocation and targeting of PKCs by blocking or inducing their interaction with anchoring proteins such as RACKs. Currently PKC-isozyme-specific inhibitor and activator peptides for all cPKCs and nPKCs are available. They have proven to be useful tools in dissecting signalling processes mediated by individual PKC isozymes [e.g. in the context of PKC effects in cardiac myocytes on contraction, ischemic cell

death, MAP kinase activation and ion channel activity (for reviews, see Csukai and Mochly-Rosen, 1999; Schechtman and Mochly-Rosen, 2002)]. Unfortunately, the use of PKC-isozyme-specific interfering peptides has been restricted to a rather small number of studies. In the majority of pharmacological studies on PKC function, the potential of the peptide interference method has been ignored, and experimental approaches have been limited to the default use of classic but often problematic pharmacological tools (i.e. phorbol esters, indolocarbazoles, and bisindolylmaleimides) described above.

An additional promising approach for interfering with PKC function involves the use of antisense oligonucleotides to knockdown PKC expression. In particular, downregulation of PKCα expression using antisense oligonucleotides appears to have significant therapeutic potential (Dean and McKay, 1994) (for reviews, see Tamm et al., 2001; Goekjian and Jirousek, 2001; Swannie and Kaye, 2002). Despite its high potential and usefulness for systematic analyses of PKC function, the antisense approach is very rarely used in basic research and largely ignored in the majority of pharmacological studies of PKC function.

In summary, the most frequently used pharmacological tools for PKC activation and inhibition (i.e. phorbol esters, indolocarbazoles and bisindolylmaleimides) are not sufficiently specific to define PKC-mediated physiological effects unequivocally in any experimental paradigm – particularly the separation of PKC-mediated effects from those caused by other C_1 domain proteins or by other kinases remains difficult. In view of the fact that most pharmacological studies of PKC function involve phorbol-ester-mediated perturbations of certain cellular parameters, followed by the addition of rather nonspecific PKC inhibitors, the involvement of alternative DAG/phorbol-ester receptors in the observed effects must be considered wherever phorbol esters are used as the main investigative tools. PKC-isozyme-specific inhibitor and activator peptides and certain antisense oligonucleoides are the most promising pharmacological tools to circumvent the problems involved in the exclusive use of phorbol esters, indolocarbazoles and bisindolylmaleimides to activate or inhibit PKCs. In addition, systematic genetic studies represent an essential experimental alternative. The fact that deletion mutations of PKCγ (Abelovic et al., 1993), PKCβ (Leitges et al., 1996), PKCε (Hodge et al., 1999; Khasar et al., 1999), PKCθ (Sun et al., 2000), PKCδ (Leitges et al., 2001a) or PKCζ (Leitges et al., 2001b) in mice have rather mild phenotypic consequences indicates that there is functional redundancy among the various PKC isozymes. To account for this problem and ultimately to determine the role of individual PKCs, multiple deletion mutations (e.g. of related PKC isozymes) may also be needed. Such genetic approaches in intact animals could then be ideally complemented with protein overexpression approaches.

Non-PKC C_1 domain proteins

Analyses of current protein sequence databases demonstrate that PKC isozymes are not the only C_1-domain-containing proteins. Indeed, the NCBI domain architecture retrieval tool (DART) identifies some 30 C_1-domain-containing protein types in mammals. Many of these lack structural features that appear to be essential for binding of DAG/phorbol ester to C_1 domains. However, apart from cPKCs and nPKCs (Sharkey and Blumberg, 1985; Zhang et al., 1995), chimaerins (Ahmed et al., 1990; Ahmed et al., 1993; Areces et al., 1994; Caloca et al., 1997; Caloca et al., 2001), PKD1 (Valverde et al., 1994), RasGRPs (Ebinu et al., 1998; Lorenzo et al., 2000), Munc13s (Betz et al., 1998) and DAG kinase γ (Shindo et al., 2001) have all been shown to bind phorbol esters and DAG with high affinity. Most of the other C_1-domain proteins identifed by the DART algorhythm have not been tested for DAG/phorbol-ester binding, and some of them (e.g. STAC, a neuron-specific SH3 domain protein) may function as DAG/phorbol-ester receptors.

Interestingly, all of the proven non-PKC DAG/phorbol-ester receptors identified here function in intracellular signalling pathways that – at least according to pharmacological evidence – are thought to be also regulated by PKCs. Such functional overlap between PKC and non-PKC DAG/phorbol-ester receptors is particularly evident in the case of the chimaerins, which are thought to play a critical role in the regulation of the actin cytoskeleton, cell cycle progression and malignant transformation (for reviews, see Ron and Kazanietz, 1999; Kazanietz, 2000; Kazanietz, 2002; Kazanietz et al., 2002). It is similarly evident in the case of RasGRPs, which function in the control of cell proliferation, differentiation and transformation by regulating the Ras/Raf/MEK/ERK pathway (Dower et al., 2000; Jones et al., 2002), and in the case of Munc13s, which are essential regulators of secretory vesicle priming and transmitter/hormone release (Betz et al., 1998; Rhee et al., 2002; for reviews, see Brose et al., 2000; Lloyd and Bellen, 2001). Given the limited specificity of the commonly used PKC-directed pharmacological tools (particularly of the universally used phorbol esters), the significant number of putative non-PKC DAG/phorbol-ester receptors and the involvement of proven non-PKC DAG/phorbol-ester receptors in cellular processes that have often been associated with PKC function in the past, it is very likely that several of the identified phorbol-ester and DAG effects in mammalian cells are in fact mediated by non-PKC DAG/phorbol-ester receptors. This view is supported by a number of studies in which pharmacological effects of phorbol esters and other PKC-directed drugs could not be correlated with PKC function (e.g. Scholfield and Smith, 1989; Fabbri et al., 1994; Simon et al., 1996; Redman et al., 1997; Stevens and Sullivan, 1998; Hori et al., 1999; Honda et al., 2000; Iwasaki et al., 2000; Waters and Smith, 2000). More recently, several studies provided direct evidence for the functional importance of the regulation of four non-PKC DAG/phorbol-ester receptors by DAG and phorbol esters in distinct cellular processes.

Functional relevance of non-PKC DAG/phorbol-ester receptors as targets of the DAG second messenger pathway

Chimaerins

Chimaerins were the first high-affinity non-PKC DAG/phorbol-ester receptors to be discovered (Hall et al., 1990; Ahmed et al., 1990). They constitute a family of two isoforms (α and β) that are expressed from different genes, and each occurs as two splice variants (1 and 2). The type 1 chimaerins contain a C_1 domain, followed by a Rac-GTPase-

activating domain, whereas the type 2 chimaerins have an additional N-terminal SH2 domain (Fig. 1) (for reviews, see Kazanietz, 2000; Kazanietz, 2002; Kazanietz et al., 2000).

In common with cPKCs and nPKCs, chimaerins translocate to phospholipid membranes in response to phorbol-ester binding (Caloca et al., 1997). Chimaerins are implicated in diverse cellular processes, such as cell adhesion (Herrera and Shivers, 1994), cytoskeletal dynamics (Herrera and Shivers, 1994), lamellipodium/filopodium formation (Kozma et al., 1996), phagocytosis (Cox et al., 1997), neuritogenesis and nerve cell development (Leung et al., 1994; Hall et al., 2001). The Rac-GTPase-activating function of chimaerins (Diekmann et al., 1991) is likely to be involved in these processes but direct evidence for a function of chimaerins in signalling to Rac in vivo is still lacking. Current information on the function of chimaerins is mostly derived from overexpression studies, and additional work using complementary methods is needed to verify an in vivo role of chimaerins in the processes mentioned above.

The C_1 domains of chimaerins are high-affinity DAG/phorbol-ester binding sites (Ahmed et al., 1990; Ahmed et al., 1993; Areces et al., 1994; Caloca et al., 1997; Caloca et al., 2001), and chimaerins act as functional phorbol-ester receptors when overexpressed in cells. β2-chimaerin, for example, translocates from a cytosolic compartment to the plasma and Golgi membranes after phorbol-ester treatment (Caloca et al., 2001; Wang and Kazanietz, 2002). This translocation is dependent on an intact C_1 domain and thought to be supported in vivo by an additional interaction with a *cis*-Golgi transmembrane protein, Tmp21-I (Wang and Kazanietz, 2002). However, phorbol esters do not (or do only very weakly) affect the GTPase-activating function of chimaerins (Ahmed et al., 1993; Kazanietz, 2002), which indicates that the function of DAG binding is primarily to translocate chimaerins to membranes, thus spatially restricting their Rac-GTPase-activating effects. How this membrane translocation of chimaerins relates to Rac signalling, particularly in the case of Golgi membranes, is unknown (Kazanietz et al., 2002).

PKD1

The PKD family consists of PKD1 (also called PKCμ), PKD2 and PKD3 (also called PKCν) (for a review, see Van Lint et al., 2002). These enzymes form a subfamily of the AGC superfamily of serine/threonine kinases that is structurally related to but distinct from other AGC superfamily members such as PKCs (Valverde et al., 1994; Nishikawa et al., 1997; Hayashi et al., 1999; Sturany et al., 2001). PKD1, the most prominent family member, contains an N-terminal apolar domain, two C_1 domains, a negatively charged central domain, a pleckstrin-homology domain and a serine/threonine kinase domain (Fig. 1). PKD1 is activated by multiple signalling mechanisms. A major PKD1 activation mechanism involves protein phosphorylation by PKCε and/or PKCη, which is likely to be triggered by G-protein-coupled receptors followed by activation of PI-PLCβ and concomitant DAG production (Iglesias et al., 1998; Matthews et al., 1999b; Vertommen et al., 2000; Waldron et al., 2001). This functional interaction between PKD1 and PKCs, which is a striking example of a mechanistic coupling between two types of DAG/phorbol-ester receptors, can be triggered in vivo by neuropeptides (via a pathway involving G-protein-coupled receptors and PI-PLCβ), growth factors (via activation of PI-PLCγ) or even oxidative stress (via Src and PI-PLCγ) (for a review, see Van Lint et al., 2002). In addition, PKD1 is regulated by 14-3-3 proteins (Hausser et al., 1999), Gβγ subunits (Jamora et al., 1999) and by caspase-mediated cleavage (Endo et al., 2000).

Although the corresponding evidence is in some cases still fragmentary and often relies only on protein overexpression studies, PKD1 is thought to be involved in the regulation of several cellular processes, including cell proliferation (Rennecke et al., 1999), cancer cell invasion of tissues (Bowden et al., 1999) and apoptosis (Johannes et al., 1998). The best characterised function of PKD1 is its regulatory role in the Golgi apparatus, where it is required for transport vesicle formation and transport of proteins from the Golgi apparatus to the plasma membrane (Jamora et al., 1999; Liljedahl et al., 2001). 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. Together with effector proteins, PKD1 then forms a vesicle budding complex that causes membrane deformation, formation of short tubules and finally vesicle fission (for a review, see Van Lint et al., 2002). Binding of DAG to the first C_1 domain of PKD1 is mainly involved in targeting and localisation of the kinase (Maeda et al., 2001; Baron and Malhotra, 2002). In addition, the C_1 domains of PKD1 may be involved in the regulation of its kinase activity (Hausser et al., 2002).

RasGRPs

RasGRPs form a family of four isoforms that are characterised by an N-terminal RasGEFN/RasGEF motif, an EF-hand motif and a C-terminal C_1 domain (Fig. 1) (Ebinu et al., 1998; Clyde-Smith et al., 2000; Rebhun et al., 2000; Lorenzo et al., 2001; Yang et al., 2002).

Through their RasGEFN/RasGEF domains, all RasGRPs act as activators of Ras and related small GTPases (Ebinu et al., 1998; Clyde-Smith et al., 2000; Rebhun et al., 2000; Lorenzo et al., 2001; Yang et al., 2002) and thus stimulate the Ras/Raf/MEK/ERK pathway. Several lines of evidence indicate that RasGRPs are involved in cell transformation (Ebinu et al., 1998; Clyde-Smith et al., 2000; Dupuy et al., 2001), T cell receptor signalling and T cell differentiation (Dower et al., 2000; Ebinu et al., 2000), and neuronal differentiation of PC12 cells (Yamashita et al., 2000). The basis of these functional roles is in most cases the Ras-activating function of RasGRPs and the resulting activation of the Ras/Raf/MEK/ERK pathway.

RasGRP1 and RasGRP3 have now been shown to be high-affinity DAG/phorbol-ester receptors (Lorenzo et al., 2000; Lorenzo et al., 2001), and RasGRP1 translocates to membrane compartments in response to phorbol-ester treatment (Ebinu et al., 1998; Tognon et al., 1998). In intact cells, RasGRP1 couples muscarinic acetylcholine receptors (Guo et al., 2001) and T cell receptors (Dower et al., 2000; Jones et al., 2002) to the Ras/Raf/MEK/ERK pathway independently of PKCs. Indeed, in Ras signalling assays and cell proliferation assays, mutant thymocytes that lack RasGRP1 are insensitive to phorbol esters and T cell receptor activation (Dower et al., 2000). These genetic data, together with evidence from complementary studies described above demonstrate

beautifully that DAG-induced induction of the Ras/Raf/MEK/ERK pathway – at least in thymocytes – is entirely dependent on RasGRP1 and unlikely to involve PKCs. This discovery is particularly striking because numerous studies in different cell types have related the activation of the Ras/Raf/MEK/ERK pathway to PKC activity – in almost all cases relying on the conventionally used pharmacological tools for PKC activation and inhibition, that is, phorbol esters, indolocarbazoles, and bisindolylmaleimides (for reviews, see Goekjian and Jirousek, 2001; Ventura and Maioli, 2001). The data obtained in RasGRP1 deleted mutant thymocytes provide the first direct and convincing evidence for a cellular DAG signalling pathway that is mediated by a non-PKC DAG/phorbol-ester receptor rather than by PKCs, as had been thought previously. It is likely that thymocytes are not the only cell type in which allegedly PKC-mediated effects on the Ras/Raf/MEK/ERK pathway are in fact caused by RasGRPs.

Munc13s

Munc13 proteins constitute a family of three mammalian homologues of *Caenorhabditis elegans* Unc-13 that are specifically localised to presynaptic active zones, the transmitter secreting compartment of neurons (Munc13-1, -2 and -3) (Brose et al., 1995; Augustin et al., 1999a; Betz et al., 1998). They are characterised by an N-terminal C_2 domain (in Munc13-1 and ubMunc13-2), a central C_1/C_2 tandem domain and a C-terminal C_2 domain (Fig. 1). In *C. elegans*, Unc-13 is essential for coordinated movement (Brenner, 1974). Functional analyses in deletion mutant mice, *C. elegans* and *Drosophila* showed that Unc-13 and Munc13s are essential for synaptic vesicle priming (Augustin et al., 1999b; Richmond et al., 1999; Aravamudan et al., 1999; Augustin et al., 2001; Varoqueaux et al., 2002). At the molecular level, Unc-13 and Munc13s act by unfolding and activating the SNARE protein syntaxin and thereby promoting SNARE complex formation (Betz et al., 1997; Brose et al., 2000; Richmond et al., 2001). In the absence of Unc-13/Munc13-mediated vesicle priming, synapses are completely unable to secrete neurotransmitter (Richmond et al., 1999; Aravamudan et al., 1999; Varoqueaux et al., 2002).

All Munc13 isoforms bind phorbol esters and DAG with high affinity and – in common with PKCs – translocate to the plasma membrane in response to phorbol-ester binding (Betz et al., 1998; Ashery et al., 2000). As is the case for PKC C_1 domains (Hommel et al., 1994; Quest et al., 1994), mutation of the first histidine residue in the Munc13-1 C_1 motif to lysine (H567K) abolishes DAG and phorbol-ester binding as well as phorbol-ester-dependent membrane translocation (Betz et al., 1998). These findings led to the hypothesis that Munc13 proteins are functional presynaptic phorbol-ester receptors and targets of the DAG second messenger pathway that act in parallel with PKCs to regulate transmitter release (Betz et al., 1998). This hypothesis conflicted with numerous pharmacological studies that had identified PKCs as the main mediators of phorbol-ester effects on transmitter release from hippocampal neurons and that had established the concept that PKCs are the only physiological DAG-dependent mediators of enhanced neurotransmitter output that have a role in transient and long-term potentiation of synaptic strength (Stevens and Sullivan, 1998) (for a review, see Majewski and Iannazzo, 1998).

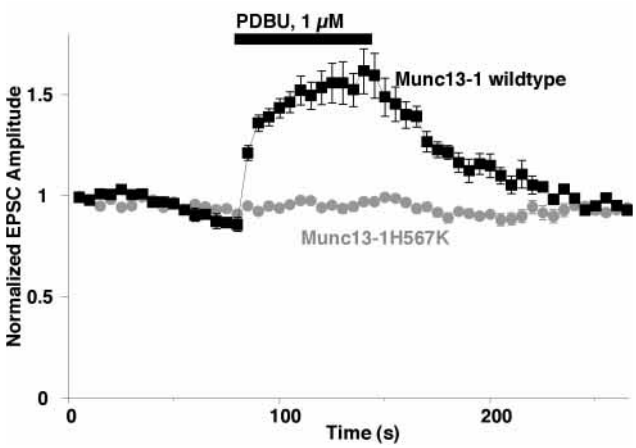

Fig. 3. Neurons expressing a phorbol-ester-binding-deficient Munc13-1 are completely insensitive to phorbol esters. Time course of phorbol-ester effects on evoked EPSCs in hippocampal neurons from wild-type-like Munc13-2-deficient mice (black squares, *n*=6) and Munc13-2-deficient/Munc13-1^{H567K} double mutant mice (grey circles, *n*=6). Application of PDBU (1 μM) is indicated by the black bar. EPSCs were evoked at 0.2 Hz and normalised to the initial amplitude. Error bars indicate s.e.m.

The functional relevance of binding of DAG/phorbol esters to Munc13-1 in vivo was determined in knockin mutant mice that express the DAG/phorbol-ester-binding-deficient *Munc13-1^{H567K}* mutant instead of the wild-type Munc13-1 from the endogenous *Munc13-1* locus (Rhee et al., 2002). Homozygous Munc13-1^{H567K} mutant mice die immediately after birth, demonstrating that an intact Munc13-1 C_1 domain is essential for survival. Hippocampal nerve cells from homozygous *Munc13-1^{H567K}* mutants are almost completely insensitive to phorbol esters, whereas wild-type cells show robust increases in transmitter release in response to phorbol-ester treatment (Rhee et al., 2002). The residual phorbol-ester sensitivity in homozygous *Munc13-1^{H567K}* cells is due to the presence of small amounts of Munc13-2, as demonstrated by the complete lack of phorbol-ester responses in cells that express *Munc13-1^{H567K}* in a *Munc13-2* deletion mutant background (Fig. 3). Because expression and function of PKCs is unaffected in *Munc13-1^{H567K}* mutants and Munc13-1 is not a substrate of phorbol-ester-activated PKCs (Rhee et al., 2002), these genetic data indicate that the phorbol-ester-induced augmentation of neurotransmitter release from hippocampal nerve cells is mediated exclusively by Munc13 proteins and not by PKCs. PKCγ, one of the prominent PKCs in synapses, has been shown not to be involved in mediating phorbol-ester effects on transmitter secretion (Goda et al., 1996). Thus, Munc13s rather than PKCs are the only functionally relevant, phorbol-ester- and DAG-sensitive presynaptic regulators of transmitter release.

In view of the *Munc13-1^{H567K}* mutant phenotype in hippocampal neurons, it is possible that other documented effects of phorbol esters on regulated secretory processes are also mediated by Munc13s rather than by PKCs. In this context, future genetic studies will have to determine whether published phorbol-ester effects on the release of catecholamines (chromaffin cells), insulin (β cells), growth hormone (pituitary), acetylcholine (neuromuscular junction) or

dopamine (striatum) are mediated by PKCs or Munc13s (Kanashiro and Khalil, 1998).

The fact that homozygous *Munc13-1*^{H567K} mutant mice die immediately after birth demonstrates that Munc13-1 – in contrast to 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 striking functional changes in hippocampal nerve cells. *Munc13-1*^{H567K} mutant cells exhibit 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 (Rhee et al., 2002). 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 appears to activate PI-PLC isozymes (e.g. PI-PLCδ and PI-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 (Rhee et al., 2002; Rosenmund et al., 2002). The fact that *Munc13-1*^{H567K} mutant mice die immediately after birth indicates that the C_1-domain-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).

A molecular model of how Munc13-1 activation by DAG regulates synaptic efficacy during periods of high synaptic activity can be inferred from the mechanism of DAG-dependent membrane recruitment of PKCs (Fig. 4). Munc13-1 is present in a soluble pool and a pool that is tightly associated with the cytoskeletal matrix of the presynaptic active zone by a proteinaceous linker (Betz et al., 2001), and soluble Munc13-1 can translocate to the plasma membrane in a phorbol-ester dependent (and presumably also DAG-dependent) manner (Betz et al., 1998; Ashery et al., 2000). The insoluble, active zone resident Munc13-1 is functionally integrated into the release machinery of the active zone, has access to all necessary regulatory proteins (Betz et al., 2001) and may define the basal pool of fusion competent vesicles that are characterised by slow pool-refilling rates and high vesicular release probability P_{vr} (Rhee et al., 2002). A second pool of fusion-competent vesicles that is dependent on the Munc13-1 C_1 domain and characterized by fast refilling rates but low P_{vr} (Rhee et al., 2002) may be generated by Munc13-1 molecules that have been recruited from the cytosol to the presynaptic plasma membrane in a DAG-dependent manner. These recruited, non-active-zone-resident Munc13-1 molecules could represent 'ectopic' priming sites that are partially functional

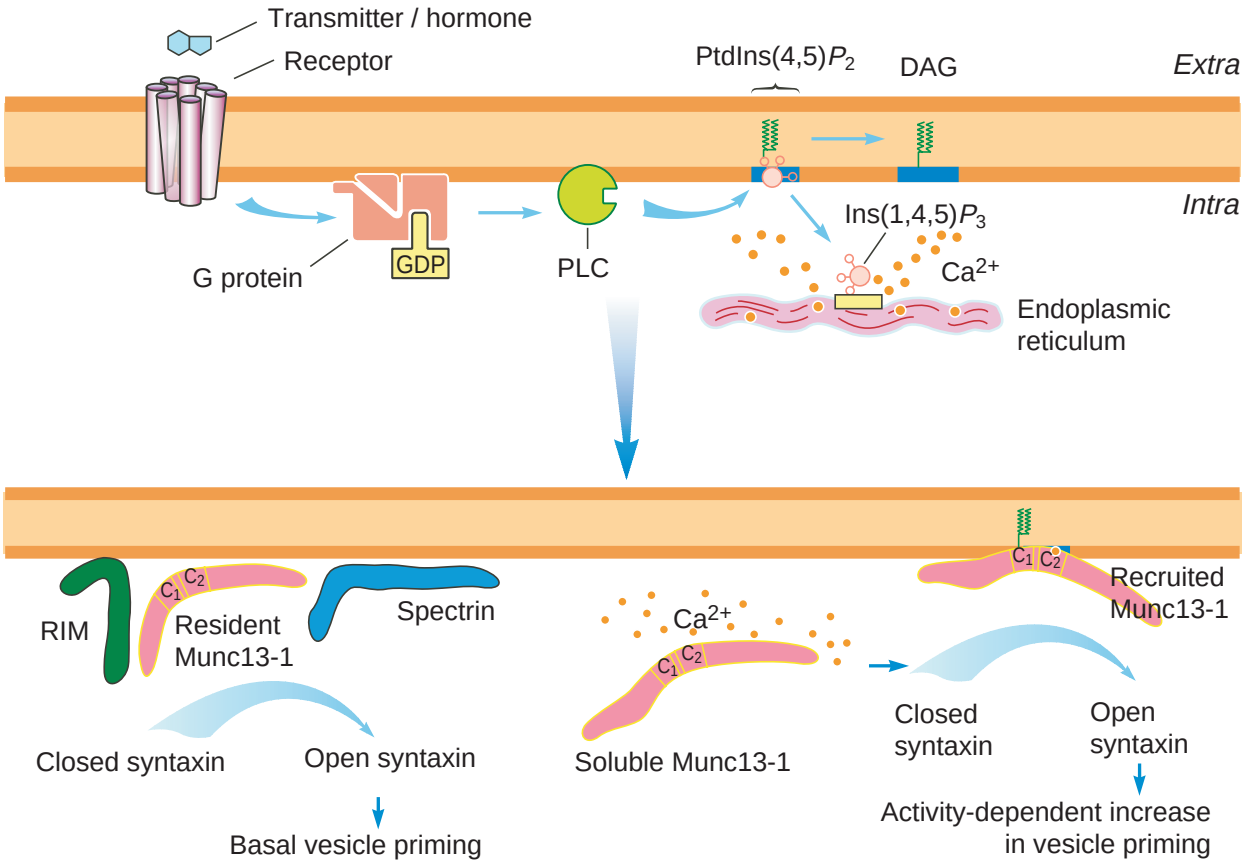

Fig. 4. Model of Munc13-1 activation by DAG. See text for details. Note that only one mechanism of DAG synthesis (i.e. PI-PLCβ) is depicted here. Other PI-PLC activities are likely to also activate Munc13s. Indeed, PI-PLCδ may be responsible for activation of Munc13-1 during high-frequency stimulation and intrasynaptic accumulation of Ca^{2+}.

but lack active-zone-specific regulatory components, hence their fast refilling rate and low P_{vr}. In the absence of stimulation, the number of 'ectopic' priming sites would depend on the resting DAG level in the presynaptic active zone plasma membrane. Tonically present 'ectopic' priming sites would be largely eliminated in *Munc13-1*^{H567K} neurons, leading to the observed reduction in the size of the readily releasable vesicle pool (Rhee et al., 2002). The number of 'ectopic' sites would be increased by activity-dependent increases in membrane DAG levels or by phorbol esters, which indeed cause increases in the size of the readily releasable vesicle pool (Stevens and Sullivan, 1998).

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, which appear to control the function of the *C. elegans* Munc13 homologue UNC-13 via $G_q\alpha$/PI-PLCβ and $G_0\alpha$/DAG kinase, respectively (Lackner et al., 1999; Miller et al., 1999; Nurrish et al., 1999). Indeed, presynaptic localisation of the soluble Unc-13 MR splice variant appears to be regulated by DAG (via the $G_0\alpha$/DAG kinase pathway) in a manner compatible with the postulated mechanism of DAG-dependent Munc13 recruitment depicted in Fig. 4 (Nurrish et al., 1999).

Conclusions

DAG is one of the most important second messengers in mammalian cells. The functionally most relevant polyunsaturated DAG species are generated from phosphatidylinositol 4,5-bisphosphate by PI-PLCs after activation of different types of cell-surface receptors. The most prominent intracellular targets of this DAG belong to the PKC family of serine/threonine kinases. However, owing to their limited specificity, the pharmacological tools that are commonly used to study PKC-mediated cellular processes (i.e. phorbol esters, indolocarbazoles and bisindolylmaleimides) are not adequate to determine the functional significance of PKC isozymes in defined cellular signalling processes. As a consequence, the involvement of PKCs in a number of allegedly PKC-mediated processes in mammalian cells remains to be proven by alternative experimental approaches. These include the systematic use of activating/inhibiting peptides, interference with PKC expression by antisense oligonucleotides or RNAi, overexpression of wild-type and dominant-interfering PKC forms and, most importantly, further genetic studies in mice. The importance of such studies is evident from work showing that mammals express at least five types of non-PKC high-affinity DAG/phorbol-ester receptor – chimaerins, PKD1, RasGRPs, Munc13s and DAG kinase γ – whose functions partially overlap with those of PKC isozymes. RasGRP1 (in the context of DAG-dependent Ras/Raf/MEK/ERK signalling) and Munc13-1 (in the context of DAG-dependent regulation of neurotransmitter release) mediate DAG/phorbol-ester effects that had previously been attributed to PKCs. This disproves the notion that all DAG/phorbol-ester effects are mediated by PKC isozymes, a view that is widely accepted in the life sciences and represents the implicit conceptual basis of most pharmacological studies on PKCs. Past and future pharmacological studies on protein kinase C must therefore be complemented with alternative experimental approaches that can distinguish between the roles of PKCs and non-PKC DAG/phorbol-ester receptors in DAG signalling processes. Detailed genetic analyses of C_1-domain-containing non-PKC DAG/phorbol-ester receptors in mammals have proven to be the most promising approach in this respect. Depending on the cellular system under investigation, RNAi is likely to be of comparable potential.

We would like to thank the members of our laboratories for discussions and invaluable contributions, in particular J.-S. Rhee and A. Betz for providing the data shown in Figs 2 and 3. Work in the authors' laboratories was generously supported by the Max-Planck-Society and the German Research Foundation.

References

Abeliovich, A., Paylor, R., Chen, C., Kim, J. J., Wehner, J. M. and Tonegawa, S. (1993). PKC γ mutant mice exhibit mild deficits in spatial and contextual learning. *Cell* **75**, 1263-1271.

Ahmed, S., Kozma, R., Monfries, C., Hall, C., Lim, H. H., Smith, P. and Lim, L. (1990). Human brain n-chimaerin cDNA encodes a novel phorbol ester receptor. *Biochem. J.* **272**, 767-773.

Ahmed, S., Lee, J., Kozma, R., Best, A., Monfries, C. and Lim, L. (1993). A novel functional target for tumor-promoting phorbol esters and lysophosphatidic acid. The p21rac-GTPase activating protein n-chimaerin. *J. Biol. Chem.* **268**, 10709-10712.

Anrather, J., Csizmadia, V., Soares, M. P. and Winkler, H. (1999). Regulation of NF-kappaB RelA phosphorylation and transcriptional activity by p21(ras) and protein kinase Czeta in primary endothelial cells. *J. Biol. Chem.* **274**, 13594-13603.

Aravamudan, B., Fergestad, T., Davis, W. S., Rodesch, C. K. and Broadie, K. (1999). *Drosophila* UNC-13 is essential for synaptic transmission. *Nat. Neurosci.* **2**, 965-971.

Areces, L. B., Kazanietz, M. G. and Blumberg, P. M. (1994). Close similarity of baculovirus-expressed n-chimaerin and protein kinase C α as phorbol ester receptors. *J. Biol. Chem.* **269**, 19553-19558.

Ashery, U., Varoqueaux, F., Voets, T., Betz, A., Thakur, P., Koch, H., Neher, E., Brose, N. and Rettig, J. (2000). Munc13-1 acts as a priming factor for large dense-core vesicles in bovine chromaffin cells. *EMBO J.* **19**, 3586-3596.

Augustin, I., Betz, A., Herrmann, C., Jo, T. and Brose, N. (1999a). Differential expression of two novel Munc13 proteins in rat brain. *Biochem. J.* **337**, 363-371.

Augustin, I., Rosenmund, C., Südhof, T. C. and Brose, N. (1999b). Munc13-1 is essential for fusion competence of glutamatergic synaptic vesicles. *Nature* **400**, 457-461.

Augustin, I., Korte, S., Rickmann, M., Kretzschmar, H. A., Südhof, T. C., Herms, J. W. and Brose, N. (2001). The cerebellum-specific Munc13 isoform Munc13-3 regulates cerebellar synaptic transmission and motor learning in mice. *J. Neurosci.* **21**, 10-17.

Baron, C. L. and Malhotra, V. (2002). Role of diacylglycerol in PKD recruitment to the TGN and protein transport to the plasma membrane. *Science* **295**, 325-328.

Barry, O. P. and Kazanietz, M. G. (2001). Protein kinase C isozymes, novel phorbol ester receptors and cancer chemotherapy. *Curr. Pharm. Des.* **7**, 1725-1744.

Betz, A., Okamoto, M., Benseler, F. and Brose, N. (1997). Direct interaction of the rat unc-13 homologue Munc13-1 with the N terminus of syntaxin. *J. Biol. Chem.* **272**, 2520-2526.

Betz, A., Ashery, U., Rickmann, M., Augustin, I., Neher, E., Südhof, T. C., Rettig, J. and Brose, N. (1998). Munc13-1 is a presynaptic phorbol ester receptor that enhances neurotransmitter release. *Neuron* **21**, 123-136.

Betz, A., Thakur, P., Junge, H. J., Ashery, U., Rhee, J.-S., Scheuss, V., Rosenmund, C., Rettig, J. and Brose, N. (2001). Functional interaction of the active zone proteins Munc13-1 and RIM1 in synaptic vesicle priming. *Neuron* **30**, 183-196.

Bowden, E. T., Barth, M., Thomas, D., Glazer, R. I. and Mueller, S. C. (1999). An invasion-related complex of cortactin, paxillin and PKCμ associates with invadopodia at sites of extracellular matrix degradation. *Oncogene* **18**, 4440-4449.

Brenner, S. (1974). The genetics of *Caenorhabditis elegans*. *Genetics* **77**, 71-94.

Brose, N., Hofmann, K., Hata, Y. and Südhof, T. C. (1995). Mammalian homologues of *C. elegans* unc-13 gene define novel family of C-domain proteins. *J. Biol. Chem.* **270**, 25273-25280.

Brose, N., Rosenmund, C. and Rettig, J. (2000). Regulation of transmitter release by Unc-13 and its homologues. *Curr. Opin. Neurobiol.* **10**, 303-311.

Caloca, M. J., Fernandez, N., Lewin, N. E., Ching, D., Modali, R., Blumberg, P. M. and Kazanietz, M. G. (1997). β2-chimaerin is a high affinity receptor for the phorbol ester tumor promoters. *J. Biol. Chem.* **272**, 26488-26496.

Caloca, M. J., Wang, H., Delemos, A., Wang, S. and Kazanietz, M. G. (2001). Phorbol esters and related analogs regulate the subcellular localization of β2-chimaerin, a non-protein kinase C phorbol ester receptor. *J. Biol. Chem.* **276**, 18303-18312.

Clyde-Smith, J., Silins, G., Gartside, M., Grimmond, S., Etheridge, M., Apolloni, A., Hayward, N. and Hancock, J. F. (2000). Characterization of RasGRP2, a plasma membrane-targeted, dual specificity Ras/Rap exchange factor. *J. Biol. Chem.* **275**, 32260-32267.

Cockcroft, S. (2001). Signalling roles of mammalian phospholipase D1 and D2. *Cell. Mol. Life Sci.* **58**, 1674-1687.

Cox, D., Chang, P., Zhang, Q., Reddy, P. G., Bokoch, G. M. and Greenberg, S. (1997). Requirements for both Rac1 and Cdc42 in membrane ruffling and phagocytosis in leukocytes. *J. Exp. Med.* **186**, 1487-1494.

Csukai, M. and Mochly-Rosen, D. (1999). Pharmacologic modulation of protein kinase C isozymes: the role of RACKs and subcellular localisation. *Pharmacol. Res.* **39**, 253-259.

Dean, N. M. and McKay, R. (1994). Inhibition of protein kinase C-α expression in mice after systemic administration of phosphorothioate antisense oligodeoxynucleotides. *Proc. Natl. Acad. Sci. USA* **91**, 11762-11766.

Dempsey, E. C., Newton, A. C., Mochly-Rosen, D., Fields, A. P., Reyland, M. E., Insel, P. A. and Messing, R. O. (2000). Protein kinase C isozymes and the regulation of diverse cell responses. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **279**, L429-L438.

Diekmann, D., Brill, S., Garrett, M. D., Totty, N., Hsuan, J., Monfries, C., Hall, C., Lim, L. and Hall, A. (1991). Bcr encodes a GTPase-activating protein for p21rac. *Nature* **351**, 400-402.

Dower, N. A., Stang, S. L., Bottorff, D. A., Ebinu, J. O., Dickie, P., Ostergaard, H. L. and Stone, J. C. (2000). RasGRP is essential for mouse thymocyte differentiation and TCR signaling. *Nat. Immunol.* **1**, 317-321.

Dupuy, A. J., Morgan, K., von Lintig, F. C., Shen, H., Acar, H., Hasz, D. E., Jenkins, N. A., Copeland, N. G., Boss, G. R. and Largaespada, D. A. (2001). Activation of the Rap1 guanine nucleotide exchange gene, CalDAG-GEF I, in BXH-2 murine myeloid leukemia. *J. Biol. Chem.* **276**, 11804-11811.

Ebinu, J. O., Bottorff, D. A., Chan, E. Y., Stang, S. L., Dunn, R. J. and Stone, J. C. (1998). RasGRP, a Ras guanyl nucleotide- releasing protein with calcium- and diacylglycerol-binding motifs. *Science* **280**, 1082-1086.

Ebinu, J. O., Stang, S. L., Teixeira, C., Bottorff, D. A., Hooton, J., Blumberg, P. M., Barry, M., Bleakley, R. C., Ostergaard, H. L. and Stone, J. C. (2000). RasGRP links T-cell receptor signaling to Ras. *Blood* **95**, 3199-3203.

Endo, K., Oki, E., Biedermann, V., Kojima, H., Yoshida, K., Johannes, F. J., Kufe, D. and Datta, R. (2000). Proteolytic cleavage and activation of protein kinase C μ by caspase-3 in the apoptotic response of cells to 1-β-D-arabinofuranosylcytosine and other genotoxic agents. *J. Biol. Chem.* **275**, 18476-18481.

Exton, J. H. (1998). Phospholipase D. *Biochim. Biophys. Acta* **1436**, 105-115.

Fabbri, M., Bannykh, S. and Balch, W. E. (1994). Export of protein from the endoplasmatic reticulum is regulated by a diacylglycerol/phorbol ester binding protein. *J. Biol. Chem.* **269**, 26848-26857.

Goda, Y., Stevens, C. F. and Tonegawa, S. (1996). Phorbol ester effects at hippocampal synapses act independently of the gamma isoform of PKC. *Learn. Mem.* **3**, 182-187.

Goekjian, P. G. and Jirousek, M. R. (2001). Protein kinase C inhibitors as novel anticancer drugs. *Expert Opin. Investig. Drugs* **10**, 2117-2140.

Goni, F. M. and Alonso, A. (1999). Structure and functional properties of diacylglycerols in membranes. *Progr. Lipid Res.* **38**, 1-48.

Guo, F. F., Kumahara, E. and Saffen, D. (2001). A CalDAG-GEFI/Rap1/B-Raf cassette couples M1 muscarinic acetylcholine receptors to the activation of ERK1/2. *J. Biol. Chem.* **276**, 25568-25581.

Hall, C., Michael, G. J., Cann, N., Ferrari, G., Teo, M., Jacobs, T., Monfries, C. and Lim, L. (2001). α2-chimaerin, a Cdc42/Rac1 regulator, is selectively expressed in the rat embryonic nervous system and is involved in neuritogenesis in N1E-115 neuroblastoma cells. *J. Neurosci.* **21**, 5191-5202.

Hall, C., Monfries, C., Smith, P., Lim, H. H., Kozma, R., Ahmed, S., Vanniasingham, V., Leung, T. and Lim, L. (1990). Novel human brain cDNA encoding a 34,000 Mr protein n-chimaerin, related to both the regulatory domain of protein kinase C and BCR, the product of the breakpoint cluster region gene. *J. Mol. Biol.* **211**, 11-16.

Hausser, A., Storz, P., Link, G., Stoll, H., Liu, Y. C., Altman, A., Pfizenmaier, K. and Johannes, F. J. (1999). Protein kinase C μ is negatively regulated by 14-3-3 signal transduction proteins. *J. Biol. Chem.* **274**, 9258-9364.

Hausser, A., Link, G., Bamberg, L., Burzlaff, A., Lutz, S., Pfizenmaier, K. and Johannes, F. J. (2002). Structural requirements for localization and activation of protein kinase C μ (PKC μ) at the Golgi compartment. *J. Cell Biol.* **156**, 65-74.

Hayashi, A., Seki, N., Hattori, A., Kozuma, S. and Saito, T. (1999). PKCν, a new member of the protein kinase C family, composes a fourth subfamily with PKCμ. *Biochim. Biophys. Acta.* **1450**, 99-106.

Herrera, R. and Shivers, B. D. (1994). Expression of alpha 1-chimaerin (rac-1 GAP) alters the cytoskeletal and adhesive properties of fibroblasts. *J. Cell. Biochem.* **56**, 582-591.

Hodge, C. W., Mehmert, K. K., Kelley, S. P., McMahon, T., Haywood, A., Olive, M. F., Wang, D., Sanchez-Perez, A. M. and Messing, R. O. (1999). Supersensitivity to allosteric GABA(A) receptor modulators and alcohol in mice lacking PKCε. *Nat. Neurosci.* **2**, 997-1002.

Hodgkin, M. N., Pettitt, T. R., Martin, A., Michell, R. H., Pemberton, A. J. and Wakelam, M. J. (1998). Diacylglycerols and phosphatidates: which molecular species are intracellular messengers? *Trends Biochem. Sci.* **23**, 200-204.

Hommel, U., Zurini, M. and Luyten, M. (1994). Solution structure of a cysteine rich domain of rat protein kinase C. *Nat. Struct. Biol.* **1**, 383-387.

Honda, I., Kamiya, H. and Yawo, H. (2000). Re-evaluation of phorbol ester-induced potentiation of transmitter release from mossy fibre terminals of the mouse hippocampus. *J. Physiol.* **529**, 763-776.

Hori, T., Takai, Y. and Takahashi, T. (1999). Presynaptic mechanism for phorbol ester-induced synaptic potentiation. *J. Neurosci.* **19**, 7262-7267.

Hurley, J. H. and Meyer, T. (2001). Subcellular targeting by membrane lipids. *Curr. Opin. Cell. Biol.* **13**, 146-152.

Iglesias, T., Waldron, R. T. and Rozengurt, E. (1998). Identification of in vivo phosphorylation sites required for protein kinase D activation. *J. Biol. Chem.* **273**, 27662-27667.

Ishaq, M., Fan, M., Wigmore, K., Gaddam, A. and Natarajan, V. (2002). Regulation of retinoid X receptor responsive element-dependent transcription in T lymphocytes by Ser/Thr phosphatases: functional divergence of protein kinase C (PKC) θ; and PKCα in mediating calcineurin-induced transactivation. *J. Immunol.* **169**, 732-738.

Iwasaki, S., Kataoka, M., Sekiguchi, M., Shimazaki, Y., Sato, K. and Takahashi, M. (2000). Two distinct mechanisms underlie the stimulation of neurotransmitter release by phorbol esters in clonal rat pheochromocytoma PC12 cells. *J. Biochem.* **128**, 407-414.

Jaken, S. and Parker, P. J. (2000). Protein kinase C binding partners. *Bioessays* **22**, 245-254.

Jamora, C., Yamanouye, N., van Lint, J., Laudenslager, J., Vandenheede, J. R., Faulkner, D. J. and Malhotra, V. (1999). Gβγ-mediated regulation of Golgi organization is through the direct activation of protein kinase D. *Cell* **98**, 59-68.

Johannes, F. J., Horn, J., Link, G., Haas, E., Siemienski, K., Wajant, H. and Pfizenmaier, K. (1998). Protein kinase Cμ downregulation of tumor-necrosis-factor-induced apoptosis correlates with enhanced expression of nuclear-factor-κB-dependent protective genes. *Eur. J. Biochem.* **257**, 47-54.

Jones, D. R., Sanjuan, M. A., Stone, J. C. and Merida, I. (2002). Expression of a catalytically inactive form of diacylglycerol kinase α induces sustained signaling through RasGRP. *FASEB J.* **16**, 595-597.

Kanashiro, C. A. and Khalil, R. A. (1998). Signal transduction by protein kinase C in mammalian cells. *Clin. Exp. Pharmacol. Physiol.* **25**, 974-985.

Kazanietz, M. G. (2000). Eyes wide shut: Protein kinase C isozymes are not the only receptors for the phorbol ester tumor promoters. *Mol. Carcinog.* **28**, 5-11.

Kazanietz, M. G. (2002). Novel 'nonkinase' phorbol ester receptors: The C1 domain connection. *Mol. Pharmacol.* **61**, 759-767.

Kazanietz, M. G., Caloca, M. J., Eroles, P., Fujii, T., Garcia-Bermejo, M. L., Reilly, M. and Wang, H. (2000). Pharmacology of the receptors for the

phorbol ester tumor promoters: multiple receptors with different biochemical properties. *Biochem. Pharmacol.* **60**, 1417-1424.

Khasar, S. G., Lin, Y. H., Martin, A., Dadgar, J., McMahon, T., Wang, D., Hundle, B., Aley, K. O., Isenberg, W., McCarter, G., Green, P. G., Hodge, C. W., Levine, J. D. and Messing, R. O. (1999). A novel nociceptor signaling pathway revealed in protein kinase C ε mutant mice. *Neuron* **24**, 253-260.

Kozma, R., Ahmed, S., Best, A. and Lim, L. (1996). The GTPase-activating protein n-chimaerin cooperates with Rac1 and Cdc42Hs to induce the formation of lamellipodia and filopodia. *Mol. Cell. Biol.* **16**, 5069-5080.

Lackner, M. R., Nurrish, S. J. and Kaplan, J. M. (1999). Facilitation of synaptic transmission by EGL-30 $G_q\alpha$ and EGL-8 PLCβ: DAG binding to UNC-13 is required to stimulate acetylcholine release. *Neuron* **24**, 335-346.

Leitges, M., Schmedt, C., Guinamard, R., Davoust, J., Schaal, S., Stabel, S. and Tarakhovsky, A. (1996). Immunodeficiency in protein kinase C β-deficient mice. *Science* **273**, 788-791.

Leitges, M., Mayr, M., Braun, U., Mayr, U., Li, C., Pfister, G., Ghaffari-Tabrizi, N., Baier, G., Hu, Y. and Xu, Q. (2001a). Exacerbated vein graft arteriosclerosis in protein kinase Cδ-null mice. *J. Clin. Invest.* **108**, 1505-1512.

Leitges, M., Sanz, L., Martin, P., Duran, A., Braun, U., Garcia, J. F., Camacho, F., Diaz-Meco, M. T., Rennert, P. D. and Moscat, J. (2001b). Targeted disruption of the ζPKC gene results in the impairment of the NF-κB pathway. *Mol. Cell* **8**, 771-780.

Leitges, M., Gimborn, K., Elis, W., Kalesnikoff, J., Hughes, M. R., Krystal, G. and Huber, M. (2002). Protein kinase C-δ is a negative regulator of antigen-induced mast cell degranulation. *Mol. Cell. Biol.* **22**, 3970-3980.

Leung, T., How, B. E., Manser, E. and Lim, L. (1994). Cerebellar β 2-chimaerin, a GTPase-activating protein for p21 ras-related rac is specifically expressed in granule cells and has a unique N-terminal SH2 domain. *J. Biol. Chem.* **269**, 12888-12892.

Liljedahl, M., Maeda, Y., Colanzi, A., Ayala, I., van Lint, J. and Malhotra, V. (2001). Protein kinase D regulates the fission of cell surface destined transport carriers from the trans-Golgi network. *Cell* **104**, 409-420.

Liscovitch, M., Czarny, M., Fiucci, G. and Tang, X. (2000). Phospholipase D: molecular and cell biology of a novel gene family. *Biochem. J.* **345**, 401-415.

Lloyd, T. E. and Bellen, H. J. (2001). pRIMing synaptic vesicles for fusion. *Nat. Neurosci.* **4**, 965-966.

Lorenzo, P. S., Beheshti, M., Pettit, G. R., Stone, J. C. and Blumberg, P. M. (2000). The guanine nucleotide exchange factor RasGRP is a high-affinity target for diacylglycerol and phorbol esters. *Mol. Pharmacol.* **57**, 840-846.

Lorenzo, P. S., Kung, J. W., Bottorff, D. A., Garfield, S. H., Stone, J. C. and Blumberg, P. M. (2001). Phorbol esters modulate the Ras exchange factor RasGRP3. *Cancer Res.* **61**, 943-949.

Maeda, Y., Beznoussenko, G. V., van Lint, J., Mironov, A. A. and Malhotra, V. (2001). Recruitment of protein kinase D to the trans-Golgi network via the first cysteine-rich domain. *EMBO J.* **20**, 5982-5990.

Majewski, H. and Iannazzo, L. (1998). Protein kinase C: A physiological mediator of enhanced transmitter output. *Prog. Neurobiol.* **55**, 463-475.

Martin, P., Duran, A., Minguet, S., Gaspar, M. L., Diaz-Meco, M. T., Rennert, P., Leitges, M. and Moscat, J. (2002). Role of ζPKC in B-cell signaling and function. *EMBO J.* **21**, 4049-4057.

Matthews, S., Iglesias, T., Cantrell, D. and Rozengurt, E. (1999a). Dynamic re-distribution of protein kinase D (PKD) as revealed by a GFP-PKD fusion protein: dissociation from PKD activation. *FEBS Lett.* **457**, 515-521.

Matthews, S. A., Rozengurt, E. and Cantrell, D. (1999b). Characterization of serine 916 as an in vivo autophosphorylation site for protein kinase D/Protein kinase Cμ. *J. Biol. Chem.* **274**, 26543-26549.

Mecklenbrauker, I., Saijo, K., Zheng, N. Y., Leitges, M. and Tarakhovsky, A. (2002). Protein kinase Cδ controls self-antigen-induced B-cell tolerance. *Nature* **416**, 860-865.

Mellor, H. and Parker, P. J. (1998). The extended protein kinase C superfamily. *Biochem. J.* **332**, 281-292.

Miller, K. G., Emerson, M. D. and Rand, J. B. (1999). Goα and diacylglycerol kinase negatively regulate the Gqα pathway in *C. elegans*. *Neuron* **24**, 323-333.

Newton, A. C. (1995). Protein kinase C: Structure, function, and regulation. *J. Biol. Chem.* **270**, 28495-28498.

Newton, A. C. (1997). Regulation of protein kinase C. *Curr. Opin. Cell. Biol.* **9**, 161-167.

Newton, A. C. (2001). Protein kinase C: structural and spatial regulation by phosphorylation, cofactors, and macromolecular interactions. *Chem. Rev.* **101**, 2353-2364.

Nishikawa, K., Toker, A., Johannes, F. J., Songyang, Z. and Cantley, L. C. (1997). Determination of the specific substrate sequence motifs of protein kinase C isozymes. *J. Biol. Chem.* **272**, 952-960.

Nurrish, S. J., Segalat, L. and Kaplan, J. M. (1999). Serotonin inhibition of synaptic transmission: GOA-1 decreases the abundance of UNC-13 at release sites. *Neuron* **24**, 231-242.

Quest, A. F. G., Bardes, E. S. G. and Bell, R. M. (1994). A phorbol ester binding domain in protein kinase Cγ. *J. Biol. Chem.* **269**, 2961-2970.

Rebhun, J. F., Chen, H. and Quilliam, L. A. (2000). Identification and characterization of a new family of guanine nucleotide exchange factors for the ras-related GTPase Ral. *J. Biol. Chem.* **275**, 13406-13410.

Redman, R. S., Searl, T. J., Hirsh, J. K. and Silinsky, E. M. (1997). Opposing effects of phorbol esters on transmitter release and calcium currents at frog motor nerve endings. *J. Physiol.* **501**, 41-48.

Rennecke, J., Rehberger, P. A., Furstenberger, G., Johannes, F. J., Stohr, M., Marks, F. and Richter, K. H. (1999). Protein-kinase-Cμ expression correlates with enhanced keratinocyte proliferation in normal and neoplastic mouse epidermis and in cell culture. *Int. J. Cancer* **80**, 98-103.

Rey, O., Sinnett-Smith, J., Zhukova, E. and Rozengurt, E. (2001). Regulated nucleocytoplasmic transport of protein kinase D in response to G protein-coupled receptor activation. *J. Biol. Chem.* **276**, 49228-49235.

Rhee, S. G. (2001). Regulation of phosphoinositide-specific phospholipase C. *Annu. Rev. Biochem.* **70**, 281-312.

Rhee, J. S., Betz, A., Pyott, S., Reim, K., Varoqueaux, F., Augustin, I., Hesse, D., Südhof, T. C., Takahashi, M., Rosenmund, C. and Brose, N. (2002). β Phorbol ester- and diacylglycerol-induced augmentation of transmitter release is mediated by Munc13s and not by PKCs. *Cell* **108**, 121-133.

Richmond, J. E., Davis, W. S. and Jorgensen, E. M. (1999). UNC-13 is required for synaptic vesicle fusion in *C. elegans*. *Nat. Neurosci.* **2**, 959-964.

Richmond, J. E., Weimer, R. M. and Jorgensen, E. M. (2001). An open form of syntaxin bypasses the requirement for unc-13 in vesicle priming. *Nature* **412**, 338-341.

Ron, D. and Kazanietz, M. G. (1999). New insights into the regulation of protein kinase C and novel phorbol ester receptors. *FASEB J.* **13**, 1658-1676.

Rosenmund, C., Sigler, A., Augustin, I., Reim, K., Brose, N. and Rhee, J. S. (2002). Differential control of vesicle priming and short-term plasticity by Munc13 isoforms. *Neuron* **33**, 411-424.

Schechtman, D. and Mochly-Rosen, D. (2002). Isozyme-specific inhibitors and activators of protein kinase C. *Methods Enzymol.* **345**, 470-489.

Scholfield, C. N. and Smith, A. J. (1989). A phorbol diester-induced enhancement of synaptic transmission in olfactory cortex. *Br. J. Pharmacol.* **98**, 1344-1350.

Schonwasser, D. C., Marais, R. M., Marshall, C. J. and Parker, P. J. (1998). Activation of the mitogen-activated protein kinase/extracellular signal-regulated kinase pathway by conventional, novel, and atypical protein kinase C isotypes. *Mol. Cell. Biol.* **18**, 790-798.

Sharkey, N. A. and Blumberg, P. M. (1985). Kinetic evidence that 1,2-diolein inhibits phorbol ester binding to protein kinase C via a competitive mechanism. *Biochem. Biophys. Res. Commun.* **133**, 1051-1056.

Shindo, M., Irie, K., Ohigashi, H., Kuriyama, M. and Saito, N. (2001). Diacylglycerol kinase γ is one of the specific receptors of tumor-promoting phorbol esters. *Biochem. Biophys. Res. Commun.* **289**, 451-456.

Simon, J. P., Ivanov, I. E., Adesnik, M. and Sabatini, D. D. (1996). The production of post-Golgi vesicles requires a protein kinase C-like molecule, but not its phosphorylating activity. *J. Cell Biol.* **135**, 355-370.

Sioud, M. and Sorensen, D. R. (1998). A nuclease-resistant protein kinase C α ribozyme blocks glioma cell growth. *Nat. Biotechnol.* **16**, 556-561.

Stevens, C. F. and Sullivan, J. M. (1998). Regulation of the readily releasable vesicle pool by protein kinase C. *Neuron* **21**, 885-893.

Sturany, S., van Lint, J., Muller, F., Wilda, M., Hameister, H., Hocker, M., Brey, A., Gern, U., Vandenheede, J., Gress, T., Adler, G. and Seufferlein, T. (2001). Molecular cloning and characterization of the human protein kinase D2. A novel member of the protein kinase D family of serine threonine kinases. *J. Biol. Chem.* **276**, 3310-3318.

Su, T. T., Guo, B., Kawakami, Y., Sommer, K., Chae, K., Humphries, L. A., Kato, R. M., Kang, S., Patrone, L., Wall, R., Teitell, M., Leitges, M., Kawakami, T. and Rawlings, D. J. (2002). PKC-β controls IκB kinase lipid raft recruitment and activation in response to BCR signaling. *Nat. Immunol.* **3**, 780-786.

Sun, Z., Arendt, C. W., Ellmeier, W., Schaeffer, E. M., Sunshine, M. J.,

Gandhi, L., Annes, J., Petrzilka, D., Kupfer, A., Schwartzberg, P. L. and Littman, D. R. (2000). PKC-θ is required for TCR-induced NF-κB activation in mature but not immature T lymphocytes. *Nature* **404**, 402-407.

Swannie, H. C. and Kaye, S. B. (2002). Protein kinase C inhibitors. *Curr. Oncol. Rep.* **4**, 37-46.

Tamm, I., Dorken, B. and Hartmann, G. (2001). Antisense therapy in oncology: new hope for an old idea? *Lancet* **358**, 489-497.

Tognon, C. E., Kirk, H. E., Passmore, L. A., Whitehead, I. P., Der, C. J. and Kay, R. J. (1998). Regulation of RasGRP via a phorbol ester-responsive C1 domain. *Mol. Cell. Biol.* **18**, 6995-7008.

Tsuru, M., Katagiri, H., Asano, T., Yamada, T., Ohno, S., Ogihara, T. and Oka, Y. (2002). Role of PKC isoforms in glucose transport in 3T3-L1 adipocytes: insignificance of atypical PKC. *Am. J. Physiol. Endocrinol. Metab.* **283**, E338-E345.

Tzivion, G., Luo, Z. and Avruch, J. (1998). A dimeric 14-3-3 protein is an essential cofactor for Raf kinase activity. *Nature* **394**, 88-92.

Valverde, A. M., Sinnett-Smith, J., van Lint, J. and Rozengurt, E. (1994). Molecular cloning and characterization of protein kinase D: a target for diacylglycerol and phorbol esters with a distinctive catalytic domain. *Proc. Natl. Acad. Sci. USA* **91**, 8572-8576.

Van Lint, J., Rykx, A., Maeda, Y., Vantus, T., Sturany, S., Malhotra, V., Vandenheede, J. R. and Seufferlein, T. (2002). Protein kinase D: an intracellular traffic regulator on the move. *Trends Cell Biol.* **12**, 193-200.

Varoqueaux, F., Sigler, A., Rhee, J. S., Brose, N., Enk, C., Reim, K. and Rosenmund, C. (2002). Total arrest of spontaneous and evoked synaptic transmission but normal synaptogenesis in the absence of Munc13-mediated vesicle priming. *Proc. Natl. Acad. Sci. USA* **99**, 9037-9042.

Ventura, C. and Maioli, M. (2001). Protein kinase C control of gene expression. *Crit. Rev. Eukaryot. Gene Expr.* **11**, 243-267.

Vertommen, D., Rider, M., Ni, Y., Waelkens, E., Merlevede, W., Vandenheede, J. R. and van Lint, J. (2000). Regulation of protein kinase D by multisite phosphorylation. Identification of phosphorylation sites by mass spectrometry and characterization by site-directed mutagenesis. *J. Biol. Chem.* **275**, 19567-19576.

Wakelam, M. J. (1998). Diacylglycerol – when is it an intracellular messenger? *Biochim. Biophys. Acta* **1436**, 117-126.

Waldron, R. T., Rey, O., Iglesias, T., Tugal, T., Cantrell, D. and Rozengurt, E. (2001). Activation loop Ser744 and Ser748 in protein kinase D are transphosphorylated in vivo. *J. Biol. Chem.* **276**, 32606-32615.

Wang, H. and Kazanietz, M. G. (2002). Chimaerins, novel non-protein kinase C phorbol ester receptors, associate with Tmp21-I (p23): evidence for a novel anchoring mechanism involving the chimaerin C1 domain. *J. Biol. Chem.* **277**, 4541-4550.

Waters, J. and Smith, S. J. (2000). Phorbol esters potentiate evoked and spontaneous release by different presynaptic mechanisms. *J. Neurosci.* **20**, 7863-7870.

Way, K. J., Chou, E. and King, G. L. (2000). Identification of PKC-isoform-specific biological actions using pharmacological approaches. *Trends Pharmacol. Sci.* **21**, 181-187.

Yamashita, S., Mochizuki, N., Ohba, Y., Tobiume, M., Okada, Y., Sawa, H., Nagashima, K. and Matsuda, M. (2000). CalDAG-GEFIII activation of Ras, R-ras, and Rap1. *J. Biol. Chem.* **275**, 25488-25493.

Yang, Y., Li, L., Wong, G. W., Krilis, S. A., Madhusudhan, M. S., Sali, A. and Stevens, R. L. (2002). RasGRP4, a new mast cell-restricted Ras guanine nucleotide-releasing protein with calcium- and diacylglycerol-binding motifs. Identification of defective variants of this signaling protein in asthma, mastocytosis, and mast cell leukemia patients and demonstration of the importance of RasGRP4 in mast cell development and function. *J. Biol. Chem.* **277**, 25756-25774.

Zhang, G., Kazanietz, M. G., Blumberg, P. M. and Hurley, J. H. (1995). Crystal structure of the cys2 activator-binding domain of protein kinase C δ in complex with phorbol ester. *Cell* **81**, 917-924.