Biochemical Pharmacology, Vol. 60, pp. 1417–1424, 2000.

ISSN 0006-2952/00/$–see front matter
PII S0006-2952(00)00470-6

COMMENTARY

Pharmacology of the Receptors for the Phorbol Ester Tumor Promoters

MULTIPLE RECEPTORS WITH DIFFERENT BIOCHEMICAL PROPERTIES

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ABSTRACT. The phorbol ester tumor promoters and related analogs are widely used as potent activators of protein kinase C (PKC). The phorbol esters mimic the action of the lipid second messenger diacylglycerol (DAG). The aim of this commentary is to highlight a series of important and controversial concepts in the pharmacology and regulation of phorbol ester receptors. First, phorbol ester analogs have marked differences in their biological properties. This may be related to a differential regulation of PKC isozymes by distinct analogs. Moreover, it seems that marked differences exist in the ligand recognition properties of the C1 domains, the phorbol ester/DAG binding sites in PKC isozymes. Second, an emerging theme that we discuss here is that phorbol esters also target receptors unrelated to PKC isozymes, a concept that has been largely ignored. These novel receptors lacking kinase activity include chimaerins (a family of Rac-GTPase-activating proteins), RasGRP (a Ras exchange factor), and Unc-13/Munc-13 (a family of proteins involved in exocytosis). Unlike the classical and novel PKCs, these "non-kinase" phorbol ester receptors possess a single copy of the C1 domain. Interestingly, each receptor class has unique pharmacological properties and biochemical regulation. Lastly, it is well established that phorbol esters and related analogs can translocate each receptor to different intracellular compartments. The differential pharmacological properties of the phorbol ester receptors can be exploited to generate specific agonists and antagonists that will be helpful tools to dissect their cellular function. BIOCHEM PHARMACOL **60**;10:1417–1424, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. phorbol esters; PKC; chimaerins; RasGRP; diacylglycerol

The phorbol esters and related diterpenes are natural products isolated from plants of the *Euphorbiaceae* and *Thymeleaceae* families, and have for many years been the preferred pharmacological tools for studying PKC,† a key molecule in the signal transduction pathways activated by growth factor- and G-protein-coupled receptors. The phorbol esters and related analogs mimic the actions of the lipid second messenger DAG, a relatively simple and highly flexible molecule generated by cellular phospholipases. The involvement of PKC in cell growth, apoptosis, and differentiation makes this molecule an attractive target for pharmacological intervention in cancer chemotherapy and other diseases. Phorbol esters have attracted great interest because of their high potency as tumor promoters in the mouse skin model of multistage carcinogenesis. After the initiation event triggered by carcinogenic agents such as DMBA, phorbol esters promote the clonal expansion of the initiated cells, leading to the development of skin papillomas and carcinomas in mice [1, 2]. The phorbol esters have become important agents for elucidating the roles of PKC in signal transduction and carcinogenesis.

The pharmacology of phorbol esters and related compounds is very complex. One of the main reasons for such complexity is the presence of multiple cellular receptors, which include several isoforms of the PKC family. Each cell type expresses multiple PKC isotypes that possess different subcellular localization, substrate specificity, and cofactor requirement. In addition, a new concept that has emerged in the last years is that phorbol esters and DAG have additional cellular targets unrelated to PKC isozymes. Therefore, it is predictable that phorbol esters activate signaling pathways that do not involve PKC. These findings suggest that the use of these agents as selective PKC activators should be re-evaluated.

A second level of complexity is the varied nature of PKC ligands. Diterpenes of the phorbol ester family as well as a large number of unrelated structural analogs can bind to PKC. These analogs include nonphorbol ester diterpenes (e.g. mezereins, octahydromezerein, and thymeleatoxin),

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† *Abbreviations*: PKC, protein kinase C; DAG, diacylglycerol; DMBA, 7,12-dimethylbenz[*a*]anthracene; PDBu, phorbol 12,13-dibutyrate; PMA, phorbol 12-myristate 13-acetate; PS, phosphatidylserine; GAP, GTPase-activating protein; MAPK, mitogen-activated protein kinase; and GFP, Green Fluorescent Protein.

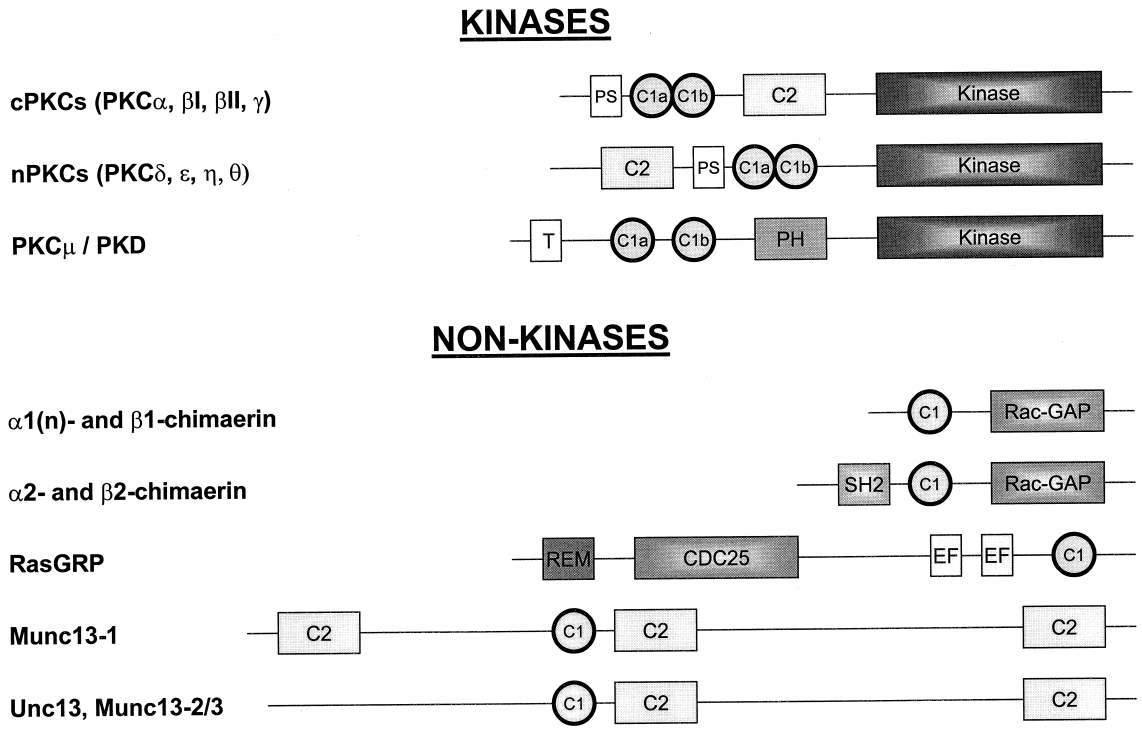

FIG. 1. Structures of phorbol ester receptors. The phorbol esters and related analogs bind to the C1 domain in PKCs and novel receptors. PS, pseudosubstrate domain; C1, cysteine-rich domain; C2, phospholipid binding domain (also a calcium binding domain in cPKCs); T, transmembrane domain, PH, PH domain; SH2, SH2 domain; Rac-GAP, Rac GTPase-activating protein domain; REM, Ras exchange motif; CDC25, region with homology to guanine exchange factor domain of Cdc25 and Sos; EF, EF hand. The aPKCs (not included in the figure) possess a single C1 domain but do not bind phorbol esters or DAG.

macrocyclic lactones (e.g. bryostatins), indole alkaloids (e.g. indolactams and teleocidins), and ingenols. PKC ligands have distinct biological and pharmacological properties, including contrasting effects in the skin tumor promotion paradigm [1, 3]. For example, unlike most potent phorbol esters, 12-deoxyphorbol esters and bryostatins are not tumor promoters but block phorbol ester-induced tumor promotion in the mouse skin [4, 5]. These effects cannot be explained by differential binding recognition properties to their cellular receptors or their intrinsic activity as PKC activators in *in vitro* assays, which suggests that other mechanisms might be involved.

PKC ISOZYMES AS PHORBOL ESTER RECEPTORS

PKC was identified in the early 1980s as the first receptor for the phorbol ester tumor promoters [6, 7]. Many laboratories, in particular that of Peter M. Blumberg at the NCI, have established the molecular basis of the interaction between phorbol esters and PKC as a receptor. An early crucial discovery was that phorbol esters and DAG bind to the same site in PKC, a finding that placed PKC as a mediator of DAG signaling after receptor activation. DAG competes with phorbol esters in binding assays using [^{3}H]PDBu as a radioligand [8]. This sensitive assay allows for the detection of specific binding with an optimal ratio of binding affinity to non-specific binding to PKC [9].

Binding of phorbol esters and DAG to PKC is dependent on phospholipids as cofactors. Acidic phospholipids fully reconstitute [^{3}H]PDBu binding, and PS is the most efficient phospholipid for reconstitution of binding activity [10, 11]. Interestingly, binding of [^{3}H]PDBu to PKC can also occur in the absence of phospholipids, although with approximately two orders of magnitude lower affinity [12]. Association with phospholipids is a key event for regulating the subcellular redistribution ("translocation") of PKCs after phorbol ester treatment or activation of receptors coupled to the generation of DAG.

PKC comprises a family of at least ten related isoforms classified into three groups: "classical" (cPKCs), "novel" (nPKCs), and "atypical" (aPKCs). The cPKC isozymes (PKCα, βI, βII, and γ) or calcium-dependent isozymes can be activated by phorbol esters/DAG and calcium. The second group or nPKCs (PKCδ, ϵ, η, and θ) are calcium-independent but can be activated by phorbol esters/DAG (Fig. 1). The aPKCs (PKCζ and ι/λ) are calcium-independent kinases that do not bind phorbol esters or DAG even with low affinity, and therefore do not belong to the phorbol ester receptor class [13–16]. A related calcium-independent kinase, termed PKCμ or PKD, has also been cloned. PKCμ (PKD) is also a DAG/phorbol ester receptor but has unique substrate specificity and regulatory mechanisms that make it a distant relative of the PKC isozymes [17, 18]. PKCν, a kinase related to PKCμ/PKD, has been

cloned recently [19]. No information exists on whether this protein is a phorbol ester receptor.

Although cPKCs have a slightly higher affinity for [^{3}H]PDBu in the presence of PS vesicles than nPKCs, the dissociation constants (K_d) for the radioligand range between 0.1 and 1 nM in all cases. However, structure–relation analysis revealed that PKC isozymes have unique patterns of ligand recognition, suggesting subtle structural differences in the binding sites. The mezerein analog thymeleatoxin is the most dramatic example of isozyme selectivity, with almost 20-fold less affinity for nPKCs than for cPKCs [20].

BINDING OF PHORBOL ESTERS AT THE CONSERVED C1 DOMAIN

All PKCs have in common a C1 domain (also known as cysteine-rich or zinc finger domain) of 50–51 amino acids located at the N-terminal regulatory region (Fig. 1). Each of these domains possesses the motif $HX_{12}CX_2CX_{13/14}CX_2CX_4HX_2CX_7C$, where H is histidine, C is cysteine, and X is any other amino acid. The C1 domain is duplicated in tandem in cPKCs and nPKCs (C1a and C1b domains), as well as in PKCμ/PKD and PKCν. A single copy is present in the phorbol ester unresponsive aPKCs [16, 19]. Through a series of deletional analysis and site-directed mutagenesis, it was established that the C1 domain is the phorbol ester binding site in cPKCs and nPKCs [12, 21–24]. This domain coordinates two Zn^{2+} atoms through cysteines and histidines that are essential for folding. Isolated C1 domains expressed as GST-fusion proteins in bacteria bind [^{3}H]PDBu in a phospholipid-dependent fashion with affinities similar to those observed with the corresponding holoenzymes [12, 22, 24].

The structure of the C1b domain of PKCδ in complex with phorbol 13-acetate has been determined by Hurley and coworkers [25]. It consists of two small β sheets and a short C-terminal α helix. The ligand binds in a groove between two pulled-apart β-sheets at the tip of the domain. Importantly, ligand binding does not induce significant changes in the conformation of the C1 domain but it creates a contiguous hydrophobic surface that promotes the insertion of the domain in the lipid bilayer. The overall structure of C1 domains of other PKCs is quite similar [26, 27]. A thorough mutational analysis of the PKCδ C1b domain confirmed the importance of hydrophobic residues for membrane insertion. The model has also been validated with indolactam analogs using computational docking studies [28].

A SINGLE C1 DOMAIN VERSUS TWO C1 DOMAINS: ARE C1 DOMAINS IN PKC EQUIVALENT?

An issue that is still controversial is that of the equivalence between C1 domains in PKCs (Fig. 2). Are both C1 domains involved in ligand binding in the holoenzyme? Do they have different ligand recognition properties? The

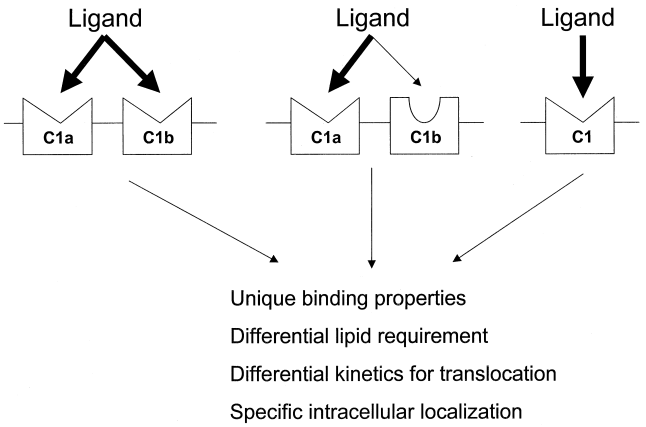

FIG. 2. Non-equivalence of C1 domains. Each C1 domain may have unique ligand-binding properties and/or differential phospholipid requirements. Phorbol esters and related analogs promote the translocation of phorbol ester receptors to different intracellular compartments, an effect that may also depend on the nature of the ligand.

stoichiometry of phorbol ester binding for intact PKC was reported to be approximately one [29]. Experiments *in vitro* using isolated C1 domains have shown contrasting results. While both C1a and C1b domains of cPKCs bind [^{3}H]PDBu with high affinity (Kazanietz MG and Blumberg PM, unpublished results), others report lack of phorbol ester binding for individual C1 domains such as the PKCδ C1a domain [30]. Moreover, a study by Irie *et al.* [31] has shown that each C1 domain of PKCγ has similar K_d values for PDBu and indolactam but differentially recognize a novel indolactam derivative, (−)-*N*13-desmethyl-*N*13-allylindolactam-G. These authors proposed that the C1a domain was the major PDBu binding site in PKCγ. Likewise, Stubbs and coworkers have determined that the PKC ligand sapintoxin D binds with distinct affinity to two binding sites in PKCα, and they observed a differential competition of binding to each site by various ligands [32, 33]. Iglesias *et al.* [34] have also proposed a differential role for C1a and C1b domains in PKCμ/PKD and suggested that the C1b domain in PKCμ/PKD is probably responsible for phorbol ester binding both *in vitro* and *in vivo*.

The non-equivalence of the C1 domains can also be observed in cellular systems. Using point mutants for each of the C1 domains of PKCδ, Szallasi *et al.* [35] reported a differential rate of translocation for each mutant after phorbol ester treatment, and suggested that the C1b domain in this nPKC may play a predominant role in translocation. A differential role for each C1 domain was also found when structure–activity for translocation was studied. The potent tumor promoters PMA and octylindolactam were selectively dependent on the C1b domain for translocation. On the other hand, this selectivity was not observed for the anti-promoters 12-deoxyphorbol esters or bryostatins, or for the second stage-tumor promoter mezerein [36]. Likewise, each C1 domain of PKCδ probably plays a different role in down-regulation mechanisms induced by bryostatins [37]. Whether these contrasting phar-

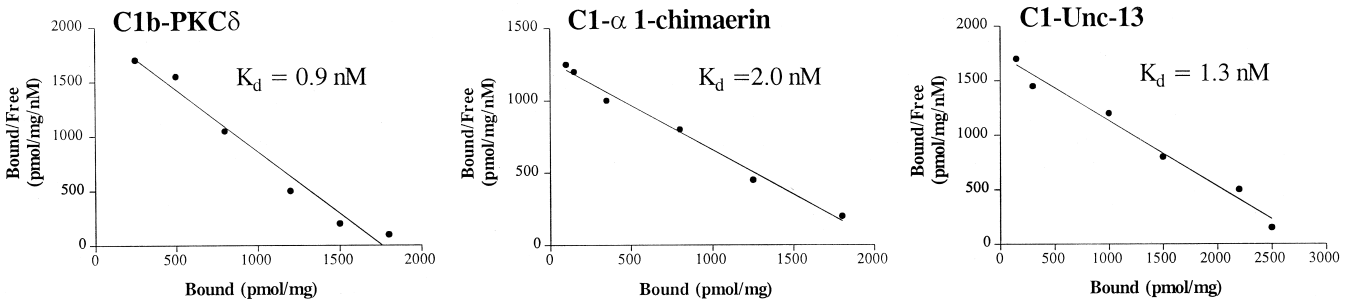

FIG. 3. Binding of [^{3}H]PDBu to GST-C1 domains of PKCδ (C1b domain), α1-chimaerin, and Unc-13. The recombinant GST-fusion proteins purified from *Escherichia coli* were assessed for [^{3}H]PDBu binding in the presence of PS vesicles. A detailed description of [^{3}H]PDBu binding assays can be found elsewhere [9, 12, 24, 52]. Each point represents the mean of three experimental values, with standard error < 2%. The dissociation constant (K_d) is the average of three experiments.

macological properties of ligands can explain their differential biology in the mouse skin is not known. Non-equivalent roles for C1 domains have also been reported in yeast proliferation assays using deletion mutants of PKCs [38]. The full consequences of the asymmetry of the PKC C1 domains are still poorly understood.

C1 DOMAINS IN NON-PKC RECEPTORS

PKCs are not the only proteins possessing C1 domains [39]. However, not all of the proteins having C1 domains are phorbol ester/DAG receptors. Interestingly, a similar overall topology was observed for C1 domains that either bind to or do not bind to phorbol esters [40]. This indeed suggests that binding properties (or lack of binding) are determined by unique specific interactions in each protein. Among the C1 domains unresponsive to phorbol esters are those of c-Raf and Vav (this last protein was mistakenly defined as a phorbol ester receptor in early papers, see Ref. 41), diacylglycerol kinases, and others [23, 39, 40].

A revolutionary finding was the discovery of novel receptors for phorbol esters and DAG that are totally unrelated to PKC isozymes. These proteins, which include chimaerins, RasGRP, *Caenorhabditis elegans* Unc-13 and mammalian Munc13s (Fig. 1), do not possess a kinase domain, and in all cases only a single copy of the C1 domain is present [16]. These observations have important implications for the study of the biology of phorbol esters because they challenge the widely accepted dogma that all phorbol ester responses proceed through the activation of PKC isozymes.

NON-KINASE PHORBOL ESTER RECEPTORS: SIMILARITIES AND DIFFERENCES

Chimaerins are GAPs for Rac, a small GTP-binding protein that plays a critical role in the regulation of actin cytoskeleton, cell cycle progression, and malignant transformation [16]. Four chimaerin isoforms (α1- or n-, α2-, β1-, and β2-chimaerin) have been isolated. The C1 domains in chimaerin isoforms possess approximately 40% homology to those in PKC isozymes. Chimaerins bind [^{3}H]PDBu (see Fig. 3) and [^{3}H]bryostatin 1 with affinities in the same low nanomolar range as cPKCs and nPKCs [42, 43]. Phorbol ester binding to chimaerins is dependent on acidic phospholipids, as previously described for PKC isozymes [42, 43]. Site-directed mutagenesis of a critical cysteine in β2-chimaerin (Cys to Ala in position 246) completely abolishes [^{3}H]PDBu binding [44]. Interestingly, the residues forming the hydrophobic cores of the C1 domain are highly conserved in β2-chimaerin, with either identical or similar corresponding residues as in the PKCδ C1b domain. Computational docking for DAG analogs suggests similar hydrogen bonding and hydrophobic interactions for the C1 domains of β2-chimaerin and PKCδ [44].

A detailed structure–activity analysis of β2-chimaerin revealed unique binding properties for this novel phorbol ester receptor. Competition of [^{3}H]PDBu binding to β2-chimaerin and PKCα with a range of analogs known to bind and activate PKC isozymes shows striking differences. Phorbol esters, 12-deoxyphorbol esters, and mezerein show somewhat lower affinities for β2-chimaerin. The mezerein analog thymeleatoxin, a potent tumor promoter, has approximately 60-fold lower affinity for β2-chimaerin than for PKCα. These differences in binding affinities are the highest reported thus far for two individual phorbol ester receptors. In contrast, DAG analogs bind with slightly higher affinity to β2-chimaerin [43, 44]. Although it is likely that ligands can spatially accommodate into the binding groove of each C1 domain, differential interactions may occur that explain unique pharmacological properties for each receptor. Mutational and structural studies will give insight into the residues conferring these differences in ligand recognition, and will permit the rational design of selective pharmacological tools for each phorbol ester receptor type.

RasGRP is a novel phorbol ester receptor isolated by a cloning approach aimed at identifying proteins that could complement a transformation-defective allele of Ras in transformation assays. RasGRP enhances the dissociation of GDP and favors the association of GTP to Ras, leading to its activation. This nucleotide exchanging activity on Ras leads to the activation of downstream effectors of Ras

(e.g. the MAPK cascade) and malignant transformation. Interestingly, the oncogenic potential of RasGRP is dependent on its C1 domain and phorbol ester activation. In fact, deletion of the RasGRP C1 domain not only abolishes phorbol ester binding but also MAPK activation and transforming activity [45, 46].

[^{3}H]PDBu binding analysis in the presence of PS vesicles revealed a K_d of 0.6 nM for RasGRP. RasGRP is also a high-affinity receptor for the bryostatins. Competition analysis using a range of PKC ligands revealed generally similar structure–activity relations between RasGRP and PKCs. The only significant difference was found with DAGs, which bind with much higher affinity to RasGRP than to PKCs or even chimaerins. A unique characteristic of RasGRP is that [^{3}H]PDBu binding to this receptor requires much lower concentrations of acidic phospholipids than PKCs. In fact, considerable binding reconstitution could be observed even in the presence of limited amounts of lipid cofactors. Although calmodulin-like EF hands are present in RasGRP, [^{3}H]PDBu binding is independent of calcium. This is in marked contrast to the calcium-dependency observed for cPKCs [47].

The third class of "non-kinase" phorbol ester receptors includes the *C. elegans* protein Unc-13 and its related human homologs Munc13–1, Munc13–2, and Munc13–3 [16, 48, 49]. These large proteins of approximately 200 kDa act as scaffold structures for numerous exocytotic proteins, including syntaxin, Doc2, and synaptotagmin [50]. Munc13 is also a pro-apoptotic protein when expressed in kidney cells [51]. Unc-13 and Munc13 isozymes possess a single C1 domain with a consensus motif identical to those of PKCs, chimaerins, and RasGRP. Unc-13 binds [^{3}H]PDBu (Fig. 3) and [^{3}H]bryostatin 1 with low nanomolar affinity. Only small differences in binding affinities compared to PKCδ were observed when different ligand classes were evaluated in [^{3}H]PDBu competition assays. Contrary to RasGRP, Unc-13 requires higher concentrations of PS for reconstitution of binding [52].

A putative phorbol ester receptor is CalDAG-GEF-1, a Rap guanine nucleotide exchange factor. This protein possesses a single C1 domain and also has an EF hand probably involved in calcium binding. Although the properties of CalDAG-GEF-1 as a phorbol ester receptor have not been studied, it was reported that PMA enhances its nucleotide exchange activity in 293T cells [53]. However, direct binding of phorbol ester and mutagenesis studies have yet to be done in order to define this protein as a phorbol ester receptor.

DIFFERENTIAL TRANSLOCATION OF PHORBOL ESTER RECEPTORS

Upon translocation to membranes by phorbol esters or by receptor stimulation, PKC isozymes are activated and transfer phosphate from ATP to specific cellular protein acceptors. Association of PKC to membranes by prolonged activation with phorbol esters and related analogs results in the exposure of proteolytic cleavage sites and down-regulation of phorbol ester responsive PKCs.

Specificity in function of PKC isozymes is determined in large by their unique subcellular localization, a process that is probably dictated by specific protein–protein interactions [13, 16, 54]. Upon PMA treatment, PKC isozymes translocate to different subcellular compartments, including not only the plasma membrane but also the nuclear membrane, different organelles, and cytoskeletal structures, depending on the cell type [13, 16, 55–57]. An important recent observation is that different PKC ligands can redistribute PKCs to different subcellular compartments, as Wang *et al.* [57] have recently demonstrated using a GFP-tagged PKCδ. For example, whereas the tumor promoters PMA and 12-deoxyphorbol 13-tetradecanoate induce the redistribution of GFP-PKCδ to the plasma membrane followed by slower nuclear membrane translocation, the inhibitors of tumor promotion bryostatin 1 and 12-deoxyphorbol 13-phenylacetate predominantly induce translocation to the nuclear membrane. A constrained DAG analog (B8-DL-B8), on the other hand, promotes translocation of GFP-PKCδ to the Golgi apparatus. A follow-up paper by the same authors has provided compelling evidence that the nature of the intracellular localization of GFP-PKCδ depends on the length of the acyl chain of the phorbol ester [58]. It is remarkable that PKCδ translocates to other cellular compartments in other cell types, i.e. to mitochondria in mouse keratinocytes [56]. These findings have several important implications. First, hydrophobic interactions of the phorbol esters may be critical in determining subcellular localization and biological activity of PKC isozymes. Second, distinct protein–lipid and protein–protein interactions in each cellular environment are probably crucial in determining localization, and may explain distinct cellular functions of a single PKC isozyme in different cell types. Lastly, these findings have important pharmacological implications. Misplacing PKC isozymes in cells by preventing the access of the kinase to their physiological substrates and/or regulatory proteins may represent a novel mechanism of selective PKC antagonism.

Like cPKCs and nPKCs, novel "non-kinase" phorbol ester receptors are also subject to subcellular redistribution by phorbol esters. PMA induces translocation of chimaerins, RasGRP, and Munc13 as judged by subcellular fractionation analysis and fluorescent microscopy. β2-Chimaerin translocates from the cytoplasm to a perinuclear region in COS-1 cells after treatment with PMA or the DAG analog B8-DL-B8. Kinetic analysis of translocation reveals that β2-chimaerin translocates at a slower rate than PKCα. Inactive phorbol ester analogs (such as 4α-PMA) or the weak β2-chimaerin ligand thymeleatoxin do not induce translocation of this novel phorbol ester receptor [43, 44]. Contrary to the differential translocation observed for GFP-PKCδ, translocation of GFP-β2-chimaerin to the perinuclear region occurs with all agonists tested thus far, including 12-deoxyphorbol esters, bryostatin 1, indole alkaloids, and diacylglycerols (Caloca MJ and Kazanietz MG,

unpublished results). RasGRP translocates to the plasma membrane in response to PMA. It is predictable that the C1 domain in RasGRP favors its membrane association and Ras activation [45, 46]. Human Munc13 is a cytoplasmic protein and is translocated to the Golgi apparatus after phorbol ester stimulation [51]. As expected, deletion of the C1 domain in the novel phorbol ester receptors abrogates phorbol ester-induced translocation [44–46, 51].

DESIGN OF SYNTHETIC DAG ANALOGS AS PROBES FOR PHORBOL ESTER FUNCTION

An exciting novel approach aimed at designing novel PKC ligands has been developed in the laboratory of Dr. Victor Marquez at the NCI. The rationale was to identify chemical templates having the main pharmacophores of DAG constrained into rigid structures, such as lactone rings. Constraining the glycerol backbone into this type of heterocyclic ring reduces the entropic penalty associated with DAG binding, a strategy that allowed for the development of high-affinity analogs such as the previously mentioned DAG B8-DL-B8. This is a remarkable achievement because the most potent DAG lactones generated thus far have affinities three orders of magnitude higher than the widely used DAG 1-oleoyl-2-acetylglycerol, and therefore are in the same range of potency as the phorbol esters [59–61]. These cyclic DAGs also bind with high potency to β2-chimaerin and promote its subcellular distribution in cells [44].

The development of the cyclic DAG lactones represents probably the best example of a pharmacophore-guided approach for the design of templates for potent PKC ligands. Based on the structural information available from x-ray and NMR studies, it was then possible to achieve "super DAG" molecules with low nanomolar affinities by introducing branched acyl and α-alkylidene chains. These modifications were designed to achieve an optimal interaction through van der Waals contacts with highly conserved amino acids of the C1 domain and with the membrane [61].

These simple DAG analogs have phorbol ester-like effects in cells, such as inhibition of epidermal growth factor binding with potencies similar to that of PDBu. Remarkably, some of the DAG-lactones display antitumor activity when tested in the NCI *in vitro* primary screen, including effects on leukemia, colon, and breast cancer cell lines [61]. Because of the simplicity of their structure, DAG-lactones may represent potential templates for the rational synthesis of potent selective agonists for individual PKC isozymes or even for novel "non-kinase" phorbol ester receptors.

CONCLUSION

For the last two decades, the phorbol esters and related compounds have been the most common tools for the activation of PKC isozymes in cellular models. Because of the tumor promoting effects of phorbol esters, PKC has become a potential target for therapeutic intervention in neoplasia and other diseases. The observation that cPKCs and nPKCs are not the only receptors for the phorbol esters and DAG suggests that responses originally attributed to PKC may proceed through the activation of PKC-independent pathways. Therefore, one of the biggest challenges in the field is to dissect the targets involved in different phorbol ester responses. Rationale design of selective agonists (or antagonists) for each class of receptor is a key to achieve this goal.

A second important issue is the involvement of PKC isozymes in cell proliferation and tumor promotion and the use of PKC agonists to modulate such responses. The original concept was that inhibition of PKC function could be an efficient approach for cancer chemotherapy. Indeed, inhibitors of PKC kinase activity (directed towards the ATP binding site in the kinase domain) proved to be important modulators of proliferative responses and represent important tools for studying PKC function in cells. Lack of selectivity, however, is a major problem with this class of compounds [62]. The issue of whether PKC agonists or antagonists could be useful agents for cancer chemotherapy is still a subject of debate. The emerging concept is that PKC agonists may indeed have therapeutic potential. In fact, bryostatin 1, an agonist *in vitro*, is currently undergoing evaluation for a number of malignancies, including leukemias, lymphomas, and melanomas [62–64]. Moreover, recent studies have shown a therapeutic effect of PMA in patients with myelocytic leukemia [65]. The diversity of effects of PKC isozymes (including opposing roles in cell growth and apoptosis, and effects on differentiation), the paradigm of PKC activation versus PKC down-regulation, and the unique pharmacological properties of PKC ligands make PKC isozymes an attractive target for selective pharmacological exploitation in cancer chemotherapy and other diseases. Our challenge is to decipher the molecular mechanisms responsible for such a complex regulation.

This work was supported with grants from the NIH, the American Cancer Society, and the Department of Defense. M. L. G-B. and P. E. are recipients of postdoctoral fellowships from Fundacion Ramon Areces, and Conselleria de Cultura, Educacio y Ciencia, Generalitat Valenciana (Spain), respectively.

References

1. Blumberg PM, Complexities of the protein kinase C pathway. *Mol Carcinog* **4:** 339–344, 1991.
2. DiGiovanni J, Modification of multistage skin carcinogenesis in mice. *Prog Exp Tumor Res* **33:** 192–229, 1991.
3. Blumberg PM, Protein kinase C as the receptor for the phorbol ester tumor promoters: Sixth Rhoads Memorial Award Lecture. *Cancer Res* **48:** 1–8, 1988.
4. Szallasi Z, Krsmanovic L and Blumberg PM, Nonpromoting 12-deoxyphorbol 13-esters inhibit phorbol 12-myristate 13-acetate induced tumor promotion in CD-1 mouse skin. *Cancer Res* **53:** 2507–2512, 1993.
5. Hennings H, Blumberg PM, Pettit GR, Herald CL, Shores R and Yuspa SH, Bryostatin 1, an activator of protein kinase C,

inhibits tumor promotion by phorbol esters in SENCAR mouse skin. *Carcinogenesis* **8:** 1343–1346, 1987.
6. Castagna M, Takai Y, Kaibuchi K, Sano K, Kikkawa U and Nishizuka Y, Direct activation of calcium-activated, phospholipid-dependent protein kinase by tumor-promoting phorbol esters. *J Biol Chem* **257:** 7847–7851, 1982.
7. Leach KL, James ML and Blumberg PM, Characterization of a specific phorbol ester aporeceptor in mouse brain cytosol. *Proc Natl Acad Sci USA* **80:** 4208–4212, 1983.
8. Sharkey NA, Leach KL and Blumberg PM, Competitive inhibition by diacylglycerol of specific phorbol ester binding. *Proc Natl Acad Sci USA* **81:** 607–610, 1984.
9. Sharkey NA and Blumberg PM, Highly lipophilic phorbol esters as inhibitors of specific [^{3}H]phorbol 12,13-dibutyrate binding. *Cancer Res* **45:** 19–24, 1985.
10. Johnson JE, Zimmerman ML and Daleke DL, Lipid structure and not membrane structure is the major determinant in the regulation of protein kinase C by phosphatidylserine. *Biochemistry* **37:** 12020–12025, 1998.
11. Konig B, Di Nitto PA and Blumberg PM, Phospholipid and Ca^{++} dependency of phorbol ester receptors. *J Cell Biochem* **27:** 255–265, 1985.
12. Kazanietz MG, Barchi JJ Jr, Omichinski JG and Blumberg PM, Low affinity binding of phorbol esters to PKC and its cysteine-rich region in the absence of phospholipids. *J Biol Chem* **270:** 14679–14684, 1995.
13. Jaken S, Protein kinase C isozymes and substrates. *Curr Opin Cell Biol* **8:** 168–173, 1996.
14. Newton AC, Regulation of protein kinase C. *Curr Opin Cell Biol* **9:** 161–167, 1997.
15. Mellor H and Parker PJ, The extended protein kinase C superfamily. *Biochem J* **332:** 281–292, 1998.
16. Ron D and Kazanietz MG, New insights into the regulation of protein kinase C and novel phorbol ester receptors. *FASEB J* **13:** 1658–1676, 1999.
17. Valverde AM, Sinnett-Smith J, Van Lint J and Rozengurt E, 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, 1994.
18. Johannes FJ, Prestle J, Eis S, Oberhagemann P and Pfizenmaier K, PKCμ is a novel, atypical member of the protein kinase C family. *J Biol Chem* **269:** 6140–6148, 1994.
19. Hayashi A, Seki N, Hattori A, Kozuma S and Saito T, PKCν, a new member of the protein kinase C family, composes a fourth subfamily with PKCμ. *Biochim Biophys Acta* **1450:** 99–106, 1999.
20. Kazanietz MG, Areces LB, Bahador A, Mischak H, Goodnight J, Mushinski JF and Blumberg PM, Characterization of ligand and substrate specificity for the calcium-dependent and calcium-independent protein kinase C isozymes. *Mol Pharmacol* **44:** 298–307, 1993.
21. Ono Y, Fujii T, Igarashi K, Kuno T, Tanaka C, Kikkawa U and Nishizuka Y, Phorbol ester binding to protein kinase C requires a cysteine-rich zinc-finger-like sequence. *Proc Natl Acad Sci USA* **86:** 4868–4871, 1989.
22. Quest AFG, Bardes ESG and Bell RM, A phorbol ester binding domain of protein kinase Cγ. Deletion analysis of the Cys2 domain defines a minimal 43-amino acid peptide. *J Biol Chem* **269:** 2961–2970, 1994.
23. Kazanietz MG, Bustelo XR, Barbacid M, Kolch W, Mischak H, Wong G, Pettit GR, Bruns JD and Blumberg PM, Zinc finger domains and phorbol ester pharmacophore. Analysis of binding to mutated form of protein kinase C ξ and the *vav* and c-*raf* proto-oncogene products. *J Biol Chem* **269:** 11590–11594, 1994.
24. Kazanietz MG, Wang S, Milne GWA, Lewin NE, Liu HL and Blumberg PM, Residues in the second cysteine-rich region of protein kinase C δ relevant to phorbol ester binding as revealed by site-directed mutagenesis. *J Biol Chem* **270:** 21852–21859, 1995.
25. Zhang G, Kazanietz MG, Blumberg PM and Hurley JH, Crystal structure of the cys2 activator-binding domain of protein kinase Cδ in complex with phorbol ester. *Cell* **81:** 917–924, 1995.
26. Ichikawa S, Hatanaka H, Takeuchi Y, Ohno S and Inagaki F, Solution structure of cysteine-rich domain of protein kinase C alpha. *J Biochem (Tokyo)* **117:** 566–574, 1995.
27. Xu RX, Pawelczyk T, Xia T-H and Brown SC, NMR structure of a protein kinase C-γ phorbol-binding domain and study of protein-lipid micelle interactions. *Biochemistry* **36:** 10709–10717, 1997.
28. Wang S, Liu M, Lewin NE, Lorenzo PS, Bhattacharrya D, Qiao L, Kozikowski AP and Blumberg PM, Probing the binding of indolactam-V to protein kinase C through site-directed mutagenesis and computational docking simulations. *J Med Chem* **42:** 3436–3446, 1999.
29. Hannun YA, Loomis CR and Bell RM, Activation of protein kinase C by Triton X-100 mixed micelles containing diacylglycerol and phosphatidylserine. *J Biol Chem* **260:** 10039–10043, 1985.
30. Hunn M and Quest A, Cysteine-rich regions of protein kinase Cδ are functionally non-equivalent. Differences between cysteine-rich regions of non-calcium-dependent protein kinase Cδ and calcium-dependent protein kinase Cγ. *FEBS Lett* **400:** 226–232, 1997.
31. Irie K, Yanai Y, Oie K, Ishizawa J, Nakagawa Y, Ohigashi H, Wender PA and Kikkawa U, Comparison of chemical characteristics of the first and the second cysteine-rich domains of protein kinase C gamma. *Bioorg Med Chem* **5:** 1725–1737, 1997.
32. Slater SJ, Ho C, Kelly MB, Larkin JD, Taddeo FJ, Yeager MD and Stubbs CD, Protein kinase Cα contains two activator binding sites that bind phorbol esters and diacylglycerols with opposite affinities. *J Biol Chem* **271:** 4627–4631, 1996.
33. Slater SJ, Taddeo FJ, Mazurek A, Stagliano BA, Milano SK, Kelly MB, Ho C and Stubbs CD, Inhibition of membrane lipid-independent protein kinase Cα activity by phorbol esters, diacylglycerols, and bryostatin-1. *J Biol Chem* **273:** 23160–23168, 1998.
34. Iglesias T, Matthews S and Rozengurt E, Dissimilar phorbol ester binding properties of the individual cysteine-rich motifs of protein kinase D. *FEBS Lett* **437:** 19–23, 1998.
35. Szallasi Z, Bögi K, Gohari S, Biro T, Acs P and Blumberg PM, Non-equivalent roles for the first and second zinc fingers of protein kinase Cδ. Effect of their mutation on phorbol ester-induced translocation in NIH 3T3 cells. *J Biol Chem* **271:** 18299–18301, 1996.
36. Bögi K, Lorenzo PS, Szállási Z, Ács P, Wagner GS and Blumberg PM, Differential selectivity of ligands for the C1a and C1b phorbol ester binding domains of protein kinase Cδ: Possible correlation with tumor-promoting activity. *Cancer Res* **58:** 1423–1428, 1998.
37. Lorenzo PS, Bögi K, Hughes KM, Beheshti M, Bhattacharyya D, Garfield SH, Pettit GR and Blumberg PM, Differential roles of the tandem C1 domains of protein kinase C δ in the biphasic down-regulation induced by bryostatin 1. *Cancer Res* **59:** 6137–6144, 1999.
38. Shieh HL, Hansen H, Zhu J and Riedel H, Differential protein kinase C ligand regulation detected *in vivo* by a phenotypic yeast assay. *Mol Carcinog* **12:** 166–176, 1995.
39. Hurley JH, Newton AC, Parker PJ, Blumberg PM and Nishizuka Y, Taxonomy and function of C1 protein kinase C homology domains. *Protein Sci* **6:** 477–480, 1997.
40. Mott HR, Carpenter JW, Zhong S, Ghosh S, Bell RM and Campbell SL, The solution structure of the Raf-1 cysteine-

rich domain: A novel Ras and phospholipid binding site. *Proc Natl Acad Sci USA* **93:** 8312–8317, 1996.
41. Gulbins E, Coggeshall KM, Baier G, Telford D, Langlet C, Baier-Bitterlich G, Bonnefoy-Berard N, Burn P, Wittinghofer A and Altman A, Direct stimulation of Vav guanine nucleotide exchange activity for Ras by phorbol esters and diglycerides. *Mol Cell Biol* **14:** 4749–4758, 1994.
42. Areces LB, Kazanietz MG and Blumberg PM, Close similarity of baculovirus-expressed *n*-chimaerin and protein kinase Cα as phorbol ester receptors. *J Biol Chem* **269:** 19553–19558, 1994.
43. Caloca MJ, Fernandez N, Lewin NE, Ching D, Modali R, Blumberg PM and Kazanietz MG, β2-Chimaerin is a high affinity receptor for the phorbol ester tumor promoters. *J Biol Chem* **272:** 26488–26496, 1997.
44. Caloca MJ, Garcia-Bermejo ML, Blumberg PM, Lewin NL, Kremmer E, Mischak H, Wang S, Nacro K, Bienfait B, Marquez VE and Kazanietz MG, β2-Chimaerin is a novel target for diacylglycerol: Binding properties and changes in subcellular localization mediated by ligand binding to its C1 domain. *Proc Natl Acad Sci USA* **96:** 11854–11859, 1999.
45. Ebinu JO, Bottorff DA, Chan EYW, Stang SL, Dunn RJ and Stone JC, RasGRP, a Ras guanyl nucleotide-releasing protein with calcium- and diacylglycerol-binding motifs. *Science* **280:** 1082–1086, 1998.
46. Tognon CE, Kirk HE, Passmore LA, Whitehead IP, Der CJ and Kay RJ, Regulation of RasGRP via a phorbol ester-responsive C1 domain. *Mol Cell Biol* **18:** 6995–7008, 1998.
47. Lorenzo PS, Behshti M, Pettit GR, Stonne JC and Blumberg PM, The guanine nucleotide exchange factor RasGRP is a high affinity target for diacylglycerol and phorbol esters. *Mol Pharmacol* **57:** 840–846, 2000.
48. Maruyama IN and Brenner S, Phorbol ester/diacylglycerol-binding protein encoded by the *unc-13* gene of *Caenorhabditis elegans*. *Proc Natl Acad Sci USA* **88:** 5729–5733, 1991.
49. Betz A, Ashery U, Rickmann M, Augustin I, Neher E, Südhof TC, Rettig J and Brose N, Munc13–1 is a presynaptic phorbol ester receptor that enhances neurotransmitter release. *Neuron* **21:** 123–136, 1998.
50. Duncan RR, Betz A, Shipston MJ, Brose N and Chow RH, Transient, phorbol ester-induced DOC2-Munc13 interactions *in vivo*. *J Biol Chem* **274:** 27347–27350, 1999.
51. Song Y, Ailenberg M and Silverman M, Human munc13 is a diacylglycerol receptor that induces apoptosis and may contribute to renal cell injury in hyperglycemia. *Mol Biol Cell* **10:** 1609–1619, 1999.
52. Kazanietz MG, Lewin NE, Bruns JD and Blumberg PM, Characterization of the cysteine-rich region of the *Caenorhabditis elegans* protein Unc-13 as a high affinity phorbol ester receptor. Analysis of ligand-binding interactions, lipid cofactor requirements, and inhibitor sensitivity. *J Biol Chem* **270:** 10777–10783, 1995.
53. Kawasaki H, Springett GM, Toki S, Canales JJ, Harlan P, Blumenstiel JP, Chen EJ, Bany IA, Mochizuki N, Ashbacher A, Matsuda M, Housman DE and Graybiel AM, A Rap guanine nucleotide exchange factor enriched highly in the basal ganglia. *Proc Natl Acad Sci USA* **95:** 13278–13283, 1998.
54. Mochly-Rosen D and Gordon AS, Anchoring proteins for protein kinase C: A means for isozyme selectivity. *FASEB J* **12:** 35–42, 1998.
55. Goodnight JA, Mischak H, Kolch W and Mushinski JF, Immunocytochemical localization of eight protein kinase C isozymes overexpressed in NIH 3T3 fibroblasts. Isoform-specific association with microfilaments, Golgi, endoplasmic reticulum, and nuclear and cell membranes. *J Biol Chem* **270:** 9991–10001, 1995.
56. Li L, Lorenzo PS, Bogi K, Blumberg PM and Yuspa SH, Protein kinase Cδ targets mitochondria, alters mitochondrial membrane potential, and induces apoptosis in normal and neoplastic keratinocytes when overexpressed by an adenoviral vector. *Mol Cell Biol* **19:** 8547–8558, 1999.
57. Wang QJ, Bhattacharyya D, Garfield S, Nacro K, Marquez VE and Blumberg PM, Differential localization of protein kinase C δ by phorbol esters and related compounds using a fusion protein with green fluorescent protein. *J Biol Chem* **274:** 37233–37239, 1999.
58. Wang QJ, Fang T-W, Fenick D, Garfield S, Bienfait B, Marquez VE and Blumberg PM, The lipophilicity of phorbol esters as a critical factor in determining the pattern of translocation of protein kinase C δ fused to green fluorescent protein. *J Biol Chem* **275:** 12136–12146, 2000.
59. Marquez VE, Nacro K, Benzaria S, Lee J, Sharma R, Teng K, Milne GW, Bienfait B, Wang S, Lewin NE and Blumberg PM, The transition from a pharmacophore-guided approach to a receptor-guided approach in the design of potent protein kinase C ligands. *Pharmacol Ther* **82:** 251–261, 1999.
60. Nacro K, Bienfait B, Lewin NE, Blumberg PM and Marquez VE, Diacylglycerols with lipophilically equivalent branched acyl chains display high affinity for protein kinase C (PK-C). A direct measure of the effect of constraining the glycerol backbone in DAG lactones. *Bioorg Med Chem Lett* **10:** 653–655, 2000.
61. Nacro K, Bienfait B, Lee J, Han KC, Kang JH, Benzaria S, Lewin NE, Bhattacharyya DK, Blumberg PM and Marquez VE, Conformationally constrained analogues of diacylglycerol (DAG). 16. How much structural complexity is necessary for recognition and high binding affinity to protein kinase C? *J Med Chem* **43:** 921–944, 2000.
62. Caponigro F, French RC and Kaye SB, Protein kinase C: A worthwhile target for anticancer drugs? *Anticancer Drugs* **8:** 26–33, 1997.
63. Gonzalez R, Ebbinghaus S, Henthorn TK, Miller D and Kraft AS, Treatment of patients with metastatic melanoma with bryostatin-1—A phase II study. *Melanoma Res* **9:** 599–606, 1999.
64. Varterasian ML, Mohammad RM, Shurafa MS, Hulburd K, Pemberton PA, Rodriguez DH, Spadoni V, Eilender DS, Murgo A, Wall N, Dan M and Al-Katib AM, Phase II trial of bryostatin 1 in patients with relapsed low-grade non-Hodgkin's lymphoma and chronic lymphocytic leukemia. *Clin Cancer Res* **6:** 825–828, 2000.
65. Han ZT, Zhu XX, Yang RY, Sun JZ, Tian GF, Liu XJ, Cao GS, Newmark HL, Conney AH and Chang RL, Effect of intravenous infusions of 12-O-tetradecanoylphorbol-13-acetate (TPA) in patients with myelocytic leukemia: Preliminary studies on therapeutic efficacy and toxicity. *Proc Natl Acad Sci USA* **95:** 5357–5361, 1998.