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MINIREVIEW

Novel "Nonkinase" Phorbol Ester Receptors: The C1 Domain Connection

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ABSTRACT

In recent years, there have been great advances in our understanding of the pharmacology and biology of the receptors for the phorbol ester tumor promoters and the second messenger diacylglycerol (DAG). The traditional view of protein kinase C (PKC) as the sole receptor for the phorbol esters has been challenged with the discovery of proteins unrelated to PKC that bind phorbol esters with high affinity, suggesting a high degree of complexity in the signaling pathways activated by DAG. These novel "nonkinase" phorbol ester receptors include chimaerins (a family of Rac GTPase activating proteins), RasGRPs (exchange factors for Ras/Rap1), and Munc13 isoforms (scaffolding proteins involved in exocytosis). In all cases, phorbol ester binding occurs at the single C1 domain present in these proteins and, as in PKC isozymes, ligand binding is a phospholipid-dependent event. Moreover, the novel phorbol ester receptors are also subject to subcellular redistribution or "translocation" by phorbol esters, leading to their association to different effector and/or regulatory molecules. Clearly, the use of phorbol esters as specific activators of PKC in cellular models is questionable. Alternative pharmacological and molecular approaches are therefore needed to dissect the involvement of each receptor class as a mediator of phorbol ester/DAG responses.

The phorbol esters and related derivatives are the most widely used tumor promoting agents in animal models of carcinogenesis. These diterpenes have been extensively studied as ligands and activators of protein kinase C (PKC), a family of serine-threonine kinases that transduce signals upon activation of tyrosine kinase and G-protein coupled receptors. It is well established that PKC is a key mediator of growth factor, hormone, and neurotransmitter actions, and it has been implicated in the control of numerous cellular functions, including proliferation, differentiation, and apoptosis. Phorbol esters mimic the actions of diacylglycerol (DAG), a lipid second messenger generated directly by the action of phospholipase C isozymes or indirectly by the phospholipase D/phosphatidic acid (PA) pathway. The higher potency and stability of the phorbol esters compared with their corresponding DAG analogs explains the widespread use of these compounds in cellular studies (Blumberg, 1991; Kazanietz, 2000).

It has long been known that PKC is the main receptor for the phorbol ester tumor promoters. Binding of phorbol esters to PKC requires phospholipids, and acidic phospholipids are the most efficient cofactors for ligand binding. DAG or phorbol esters are required for the reversible recruiting of PKC to membranes, a process referred to as "PKC translocation". Specific modules in PKC isozymes are required for these lipid interactions as well as for protein-protein associations that regulate subcellular targeting. In this regard, the conserved C1 and C2 domains in PKC isozymes play a key role in membrane association. This review will focus on the molecular interactions between the phorbol esters and their binding site, the C1 domain. The main issue that will be discussed here is the novel concept that phorbol esters and DAG can also mediate cellular responses through the activation of proteins unrelated to PKC that possess a C1 domain. Although popular models of DAG signaling and phorbol ester

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ABBREVIATIONS: PKC, protein kinase C; DAG, diacylglycerol; PA, phosphatidic acid; cPKC, classic/conventional PKC; nPKC, novel protein kinase C; PDBu, phorbol 12,13-dibutyrate; GAP, GTPase-activating protein; PS, phosphatidylserine; GEF, guanine nucleotide exchange factor; PMA, phorbol 12-myristate 13-acetate; ERK, extracellular signal-regulated kinase; TCR, T cell receptor.

activation describe PKC as their main receptor, the involvement of novel "nonkinase" phorbol ester receptors has received considerably less attention.

Phorbol Ester Responsive and Unresponsive C1 Domains

Based on their structural and biochemical properties, PKC isozymes can be categorized into three groups, of which only two (cPKCs and nPKCs) bind phorbol esters and DAG. The classical or conventional PKCs (cPKCs) include PKCα, -βI, -βII, and -γ, which are physiologically regulated by calcium and DAG. The novel PKCs (nPKCs) include PKCδ, -ϵ, -η, and -θ. The nPKC isozymes are calcium-independent but can be activated by DAG. The atypical PKCs (PKCζ and λ/ι) are calcium-insensitive and phorbol ester/DAG-unresponsive. A related kinase, PKCμ/PKD, can also be regulated by phorbol esters, but the pattern of substrate specificity is totally different from that of PKC isozymes (Fig. 1). Details of the structural aspects of PKC isozymes and regulation of PKC function can be found in many excellent reviews published in recent years (Hurley et al., 1997; Newton and Johnson, 1998; Csukai and Mochly-Rosen, 1999; Dempsey et al., 2000; Jaken and Parker, 2000; Parekh et al., 2000; Cho, 2001).

Through a series of deletional studies and site-directed mutagenesis, it was established that the C1 domain, a motif of 50 or 51 amino acids located in the N-terminal regulatory region of PKC, is the minimum domain required for phorbol ester/DAG binding (Ono et al., 1989; Kazanietz et al., 1994, 1995a; Quest et al., 1994). This domain is duplicated in tandem (C1a and C1b) in cPKCs, nPKCs, and in PKCμ/PKD. The C1 domains are rich in cysteine and possess 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 two histidines and five of the cysteines coordinate two Zn^{2+} ions in each C1 domain. Mutation of any of the essential histidines or cysteines affects the structural integrity of the domain and consequently disrupts ligand binding (Kazanietz et al., 1995c). Important features have been revealed when the structure of the PKCδ C1b domain in complex with phorbol ester was elucidated by X-ray crystallography (Zhang et al., 1995). The domain consists of two small β sheets and a C-terminal α-helix. Phorbol esters insert lengthwise into a narrow groove between two pulled-apart β strands at one tip of the domain, and in this way form a contiguous hydrophobic surface. The acyl chain of phorbol esters is involved in the insertion of the C1 domain into the membrane. It is also recognized that hydrophobic residues at the rim of the binding cleft that are positioned toward the membrane are critical for ligand and lipid interactions (Medkova and Cho, 1999; Wang et al., 2001). Ligand binding to the C1 domain leads to a large-scale conformational change in PKC that results in the allosteric activation of the enzyme and stimulation of its phosphotransferase activity (Orr et al., 1992; Newton, 1997; Dutil and Newton, 2000).

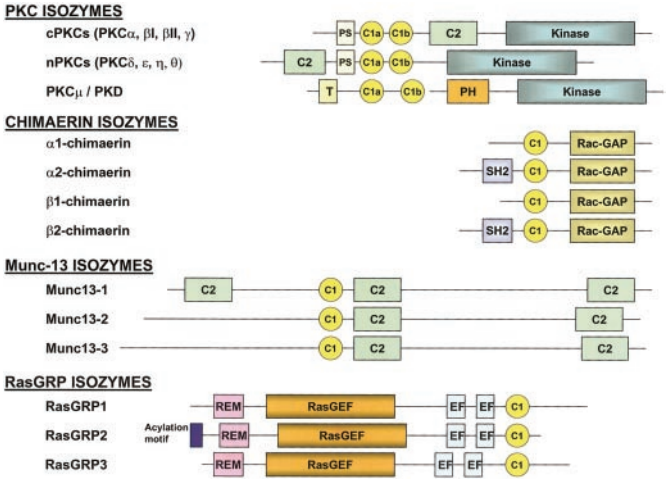

Fig. 1. Structure of PKC isozymes and novel phorbol ester receptors. PS, pseudosubstrate domain; PH, PH domain; SH2, SH2 domain; T, transmembrane domain; Rac-GAP, Rac GTPase-activating protein domain; REM, Ras exchange motif; RasGEF, region with homology to the nucleotide exchange factor domain of Sos; EF, EF hands. The C1 domain is responsible for the high-affinity binding of phorbol esters and DAG. The aPKCs (not included in the figure) possess a single C1 domain that is unable to bind DAG or phorbol esters.

Although many proteins that have C1 domains are found in databases, in most cases, these C1 domains lack essential features for phorbol ester/DAG recognition, such as in the case of PKCζ, Raf-1, DAG kinases, or Vav (this last protein was mistakenly defined as a phorbol ester receptor in earlier articles). Interestingly, a similar overall topology was observed for the phorbol ester-sensitive and phorbol ester-insensitive C1 domains, as revealed by structural studies of the Raf-1 C1 domain. However, residues that are critical for ligand binding are not present in these phorbol ester-insensitive C1 domains. For example, a loop between two β strands is absent in the Raf-1 C1 domain, and some of the essential hydrophobic residues are not present. Nevertheless, the Raf-1 C1 domain binds acidic phospholipids and is probably involved in the interaction with Ras (Mott et al., 1996), suggesting that phorbol ester-unresponsive C1 domains are still implicated in lipid- and/or protein-protein associations.

One of the important novel concepts that emerged in the past few years is that C1 domains of proteins unrelated to the PKC isozymes are capable of binding phorbol esters with high affinity (Ron and Kazanietz, 1999; Kazanietz, 2000). A key finding was the discovery of *n*-chimaerin, a protein unrelated to PKCs that has a phorbol ester-responsive C1 domain (Hall et al., 1990). When expressed in *Escherichia coli* as a TrpE- or GST-fusion protein, recombinant *n*-chimaerin bind [^{3}H]phorbol 12,13-dibutyrate (PDBu) after renaturation in the presence of Zn^{2+}. Although the initial binding study revealed a dissociation constant (K_d) for [^{3}H]PDBu higher than those reported for PKC isozymes (Ahmed et al., 1990), a subsequent characterization of this protein revealed affinities for phorbol esters and DAG that were indistinguishable from those of PKCs (Areces et al., 1994). Several additional proteins possessing a single C1 domain were later defined as phorbol ester receptors: *Caenorhabditis elegans* Unc-13 and its mammalian homologs, the Munc13s (Maruyama and Brenner, 1991; Brose et al., 1995) and mammalian RasGRP (Ebinu et al., 1998). Pharmacological characterization of these proteins indicates that they all have the structural elements required for phorbol ester binding within the C1 domain. The alignment of phorbol ester-responsive C1 domains is shown in Fig. 2. A unique feature of these novel phorbol ester receptors is that, unlike PKC isozymes, they do not have a kinase domain in their structure. These findings raised the hypothesis that DAG signaling may proceed through alternative, PKC-independent pathways.

Ligand Binding Properties of the Novel Phorbol Ester Receptors

The structure of the novel "non-PKC" phorbol ester receptors is shown in Fig. 1. Based on the experimental evidence collected in the last years, it is now clear that all phorbol ester receptors bind their ligands with high affinity, although marked differences in structure-activity and lipid-cofactor requirements exist among them. In the following sections, a detailed characterization of the novel "nonkinase" phorbol ester receptors is presented.

Pharmacological Properties of Chimaerins. This novel family of phorbol ester receptors resembles a "chimaera" between the regulatory region of PKC isozymes and BCR, the breakpoint cluster region protein involved in the translocation of Philadelphia chromosome in chronic myelogenous leukemia. *n*-Chimaerin (later renamed α1-chimaerin) was originally isolated as a 34-kDa protein highly expressed in brain (Hall et al., 1990). Three additional isoforms (α2-, β1-, and β2-chimaerin) were isolated later, all of which had a single C1 domain highly homologous to C1 domains in PKC isozymes (see Fig. 2). These proteins are alternative spliced products from the α- and β-chimaerin genes. Because splicing occurs upstream of the C1 domain, products from each gene have identical C1 domains (Hall et al., 1993; Leung et al., 1993, 1994). The C1 domains of α- and β-chimaerins are almost identical (94% identity). The C-terminal breakpoint cluster region homology domain of *n*-chimaerin has GTPase-activating protein (GAP) activity for Rac and therefore promotes the hydrolysis of GTP to GDP from this small GTP-binding protein (Diekmann et al., 1991). The main structural difference between the spliced variants is an SH2 domain located at the N terminus of α2- and β2-chimaerins (Fig. 1).

A thorough characterization of β2-chimaerin as a phorbol ester receptor showed important similarities with PKC isozymes and also striking differences. Scatchard plot analysis revealed that β2-chimaerin binds [^{3}H]PDBu with high affinity in the presence of phosphatidylserine (PS) vesicles. The K_d value is approximately 1 nM (Caloca et al., 1997), which is in the same range as the K_d values of cPKCs and nPKCs for this radioligand (Kazanietz et al., 1993). Contrasting results were observed when structure-activity relationship was studied. The most remarkable difference was found for the ligand thymeleatoxin, an analog of the second-stage tumor promoter mezerein. This ligand showed a marked preference for PKCα relative to β2-chimaerin (approximately 60-fold). This difference in ligand binding affinity is the greatest observed so far for different phorbol ester receptor classes using in vitro assays (Caloca et al., 1997). On the other hand, several DAG analogs show a slight preference for β2-chimaerin relative to PKCα (Caloca et al., 1999). Studies of cofactor dependence show that PS is the most effective phospholipid for supporting [^{3}H]PDBu binding. Unlike PKCα, PS dependence and ligand binding affinity were not affected by calcium; in this regard, β2-chimaerin resembles the nPKCs (Caloca et al., 1997). These results lead to several important conclusions. First, whereas ligands can spatially accommodate into the binding groove of the β2-chimaerin C1 domain, it is likely that unique interactions with specific residues take place within each C1 domain. Subtle structural differences between C1 domains might exist to explain differences in ligand recognition. Second, differences observed in cofactor-dependence, namely calcium and/or lipid requirement, may confer unique regulatory properties to each phorbol ester receptor in a cellular context and probably contribute to their differential intracellular targeting. Lastly, differences in binding properties may have important implications for the selective pharmacological manipulation of each receptor class.

h α1/ α2-chimaerin	HNFKVHTFRGPHWCEYCANFMWGLIAQGVKCADCGLNVHKQCSKMVPNDC
h β1/ β2 -chimaerin	HNFKVHTFRGPHWCEYCANFMWGLIAQGVRCSDCGLNVHKQCSKHVPNDC
h Mmunc13	HNFEVWTATTPTYCYECEGLLWGIARQGMRCSECGVKCHEKCQDLLNADC
r Munc13-1	HNFEVWTATTPTYCYECEGLLWGIARQGMRCTECGVKCHEKCQDLLNADC
r Munc13-2	HNFEVWSATTPTYCYECEGLLWGLARQGMRCSECGVKCHEKCQDLLNADC
r Munc13-3	HNFEVWTATTPTYCYECEGLLWGIARQGMKCLECGVKCHEKCQDLLNADC
h RasGRP1	HNFQETTYLKPTFCDNCAGFLWGVIKQGYRCKDCGMNCHKQCKDLVVFEC
h RasGRP2	HNFQESNSLRPVACRHCKALILGIYKQGLKCRACGVNCHKQCKDRLSVEC
h RasGRP3	HNFQEMTYLKPTFCEHCAGFLWGIIKQGYKCKDCGANCHKQCKDLLVLAC
Consensus	H F P ▼C C ▲▲WG▲ QG▲■C □CG H C ▲ C
h PKCδC1b	HRFKVHNYMSPTFCDHCGSLLWGLVKQGLKCEDCGMNVHHKCREKVANLC

Fig. 2. Alignment of the C1 domains of novel phorbol ester receptors. The C1 domain of RasGRP2 has been included even if there is no evidence yet that it binds phorbol esters. The PKCδ C1b domain is included for comparison. Conserved residues are shown in bold. ▼, aromatic amino acid; ▲, hydrophobic amino acid (I, L, V, M); ■, basic amino acid (K, R); □, acidic amino acid (D, E). *h*, human; *r*, rat.

Pharmacological Properties of Unc-13 and Munc13 Isoforms. Unc-13 was identified in a search for genes responsible for defects in coordinated movement in *C. elegans* and encodes a 1734-amino acid protein with sequence similarity to the regulatory region of PKC (Maruyama and Brenner, 1991). The central region of Unc-13 has a single C1 domain and a C2 domain located immediately downstream. A second C2 domain is located at the carboxyl terminus. As in the nPKCs, the C2 domains in Unc-13 are involved in phospholipid recognition in a calcium-independent manner. Although the initial characterization of this protein shows that it binds [^{3}H]PDBu in a phospholipid-independent manner, subsequent reports revealed that, as expected, ligand binding was phospholipid-dependent (Ahmed et al., 1992, Kazanietz et al., 1995b). Scatchard plot analysis using [^{3}H]PDBu showed a low nanomolar affinity for the C1 domain of Unc-13 expressed in *E. coli*, and only modest differences in ligand recognition were observed compared with PKCδ (Kazanietz et al., 1995b).

Homologs of Unc-13 in mammalian (Munc13) and *Drosophila melanogaster* (Dunc13) have been isolated (Brose et al., 1995; Aravamudan et al., 1999; Song et al., 1999). Three mammalian isoforms exist: Munc13-1, Munc13-2, and Munc13-3. These are large, brain-specific proteins with divergent N termini and conserved C termini containing C1 and C2 domains. A third C2 domain is present only at the N-terminal region of Munc13-1, suggesting potential differences in phospholipid regulation between Munc13 isoforms (Fig. 1). Experiments using a GST-fused C1 domain of Munc13-1 revealed that it binds [^{3}H]PDBu with high affinity (K_d = 5 nM using liposomes containing 20% of PS), and mutation of one of the essential histidines within the C1 domain abolished ligand binding. As observed for phorbol ester responsive PKCs, DAG displaces [^{3}H]PDBu from the Munc13-1 binding site (Betz et al., 1998). There is not yet any evidence that the *D. melanogaster* homolog binds phorbol esters.

Pharmacological Properties of RasGRPs. RasGRP1 (originally named RasGRP) is the prototype of a novel family of guanine nucleotide exchange factors (GEFs), enzymes that

catalyze the exchange of GDP by GTP in GTP-binding proteins and thereby promote their activation. RasGRP1 was identified by Stone and coworkers using a fibroblast transformation assay in a search for proteins that could complement a transformation-defective allele of Ras (Ebinu et al., 1998). This protein is highly expressed in brain and thymus and is also found in bone marrow, spleen, and kidney (Ebinu et al., 1998; Kawasaki et al., 1998; Tognon et al., 1998; Yamashita et al., 2000). Sequence analysis shows a single C1 domain located at the C-terminal region. RasGRP1 also possesses a pair of atypical EF hands that bind calcium; a proline-rich motif; and the domains responsible for nucleotide exchange, the CDC25 box and the Ras exchange motifs (Ebinu et al., 1998; Tognon et al., 1998).

[^{3}H]PDBu binds to the RasGRP1 C1 domain with an affinity of 0.6 nM in the presence of PS vesicles. Structure-activity analysis reveals only minor differences in ligand recognition compared with PKCs. However, RasGRP1 has distinct lipid cofactor dependence, as described recently by Lorenzo et al. (2000). Indeed, the C1 domain plus the EF hand motif was markedly less dependent on acidic phospholipids than PKCα. Despite the presence of the atypical EF hands, phorbol ester binding was not affected by calcium.

Related RasGRPs have been recently isolated (Fig. 1). CalDAG-GEF-I (also called GRP2 or HCDC25L) is a GEF for Rap1 (Kawasaki et al., 1998; Rebhun et al., 2000). Its alternatively spliced variant, RasGRP2, has GEF activity for Rap1, N-Ras, and K-Ras (Clyde-Smith et al., 2000). So far, there is no evidence that RasGRP2 variants bind phorbol esters or DAG. A third member of the group is RasGRP3, a Ras exchange factor (Rebhun et al., 2000; Yamashita et al., 2000). Recent evidence from the Blumberg's lab shows that RasGRP3 is also a high affinity phorbol ester receptor in the presence of anionic phospholipids. The K_d value of [^{3}H]PDBu for RasGRP3 in the presence of PS vesicles is 1.5 nM (Lorenzo et al., 2001).

Regulation and Function of the Novel Phorbol Ester Receptors

The accepted model for the regulation of PKC activity involves a conformational change and allosteric activation upon DAG/phorbol ester binding. One of the hallmarks for the activation of PKC isozymes by phorbol esters is their translocation or change in intracellular localization, a complex process that depends on lipid-binding modules. After engagement of the C1 and C2 domains to the membrane, the autoinhibitory pseudosubstrate in PKC is removed from the substrate-binding site in the catalytic region, as demonstrated in a series of elegant studies by the Newton lab (Orr et al., 1992; Orr and Newton, 1994; Dutil and Newton, 2000). PKC translocation also involves a series of protein-protein interactions that play an important role in determining intracellular localization as well as integration with other signaling pathways, thereby conferring function specificity for each PKC isozyme (Mochly-Rosen and Gordon, 1998; Ron and Kazanietz, 1999; Jaken and Parker, 2000).

Considering that the novel phorbol ester receptors have only a single C1 domain and in most cases lack other phospholipid-interacting motifs present in PKCs, a key question was whether they are able to redistribute in response to phorbol ester stimulation. In this regard, strong experimental evidence indicates that chimaerins, Munc13s and RasGRPs redistribute in response to phorbol esters. The differential localization of each novel receptor and the functional consequences of such redistribution will be discussed in the next sections.

Activation of Chimaerins by Phorbol Esters: Rac Regulation and an Important GAP. The first novel phorbol ester receptor shown to translocate in response to phorbol esters was β2-chimaerin. Using subcellular fractionation techniques in COS-1 cells, Caloca et al. (1997) found that PMA redistributes this Rac-GAP protein from the soluble (cytosolic) to a particulate fraction. However, remarkable differences in the kinetics of translocation and dose-dependence exist between β2-chimaerin and PKCα. The looser membrane association found for β2-chimaerin may indeed reflect the absence of some of the essential structural motifs present in PKC isozymes. In addition, molecular modeling studies revealed that a positively charged amino acid in the β2-chimaerin C1 domain (arginine in position 9 of the motif) makes the surface less hydrophobic and thus intrinsically less capable of membrane association (Fig. 3). Structure-activity analysis for translocation shows that thymeleatoxin, a poor ligand for chimaerins, failed to translocate β2-chimaerin even though it potently redistributes PKCα. Reduced hydrogen bonding interactions with the hydrophobic core in the β2-chimaerin C1 domain may contribute to the inability of this ligand to redistribute β2-chimaerin (Caloca et al., 2001).

Phorbol ester-induced translocation of β2-chimaerin was found to be independent of PKC activation, because it still occurs in the presence of a PKC inhibitor. Moreover, disruption of the β2-chimaerin C1 domain by mutation of essential

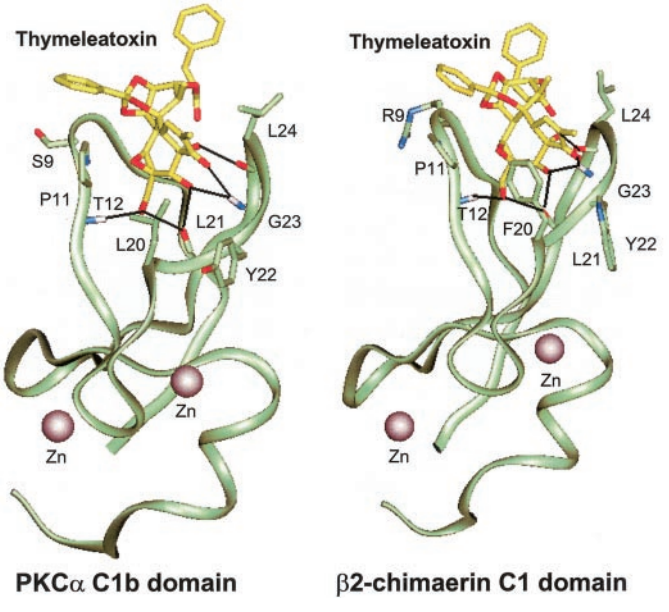

Fig. 3. Modeling of the PKCα C1b and β2-chimaerin C1 domains. Phorbol ester and related analogs bind at the tip of the domain and create a contiguous hydrophobic surface that promotes the insertion of the domain in the lipid bilayer. The figure shows the docking of thymeleatoxin to each C1 domain. [Reprinted from Caloca MJ, Wang HB, Delemos A, Wang S, and Kazanietz MG (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. Copyright © 2001 American Society for Biochemistry and Molecular Biology. Used with permission.]

cysteine 246 (the third cysteine in the motif) abolished translocation (Caloca et al., 1999). The requirement of the β2-chimaerin C1 domain for translocation was confirmed by deletional analysis (Caloca et al., 2001). More importantly, these results support the concept that a single C1 domain is sufficient for translocation, as described previously in experiments using isolated PKC C1 domains (Oancea et al., 1998) and mutated PKC isozymes (Szallasi et al., 1996; Bogi et al., 1998; Lorenzo et al., 1999).

Studies using GFP-β2-chimaerin revealed a cytoplasmic staining in the absence of phorbol ester stimulation, and a significant translocation both to the plasma membrane and to the perinucleus after phorbol ester treatment (Fig. 4). Interestingly, colocalization of β2-chimaerin with a Golgi marker was observed (Caloca et al., 2001; Wang and Kazanietz, 2002). Early experiments using PKCε mutants show that the C1 domain probably plays a role in Golgi targeting (Lehel et al., 1995, 1996). More recently, Maeda et al. (2001) reported that the C1a domain of PKCμ/PKD recruits this PKC-related kinase to the Golgi. In a search for chimaerin-interacting proteins that may be involved in perinuclear targeting, we have recently isolated Tmp21-I, a *cis*-Golgi protein. Tmp21-I is a member of the p24 family of transmembrane proteins involved in sorting/trafficking in the early secretory pathway. Deletion of the C1 domain in either α1-chimaerin or β2-chimaerin impairs the interaction, thereby implying a novel function for this domain in protein-protein associations in addition to its role in lipid and phorbol ester binding. A remarkable finding is that PMA is capable of promoting the association of β2-chimaerin with Tmp21-I at the Golgi in a PKC-independent manner, which supports a functional role of Tmp21-I as a chimaerin anchoring protein (Wang and Kazanietz, 2002). Therefore, in analogy to PKC isozymes, association with specific interacting proteins may also play a role in the intracellular targeting of chimaerins. Although very little information is available on the regulation of Golgi function and intracellular transport mechanisms by phorbol ester receptors, a role for DAG in protein transport from the Golgi to the cell surface has been described previously (Huijbregts et al., 2000). Interestingly, α1-chimaerin regulates Golgi stability during interphase (Alonso et al., 1998).

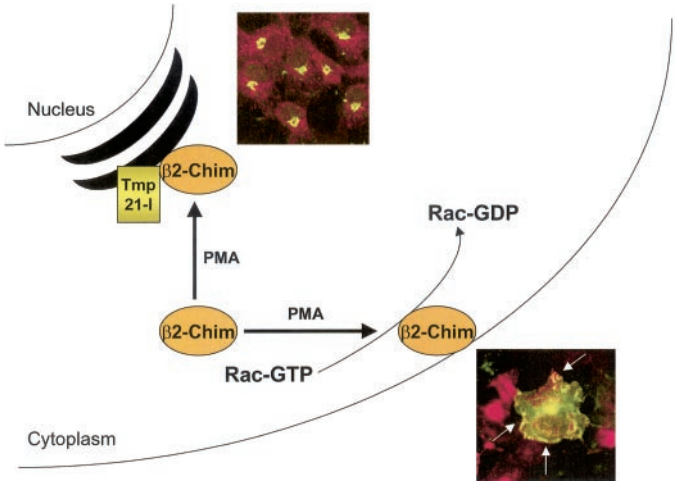

Fig. 4. Translocation of β2-chimaerin. Phorbol esters promote a dual translocation of β2-chimaerin to the plasma membrane and the perinuclear region. At the plasma membrane, β2-chimaerin associates with Rac and promotes the hydrolysis of GTP from this GTPase, leading to its inactivation. At the perinuclear site, β2-chimaerin and other chimaerin isoforms bind to Tmp21-I, which probably serves as a chimaerin-anchoring protein. Top, colocalization (*yellow*) by fluorescent microscopy of β2-chimaerin (*red*) and Tmp21-I (*green*) in the perinuclear region of COS-1 cells. Bottom, colocalization (*yellow*) of β2-chimaerin (*green*) and RacV12 (*red*) in the plasma membrane of COS-1 cells, predominantly in membrane ruffles, as indicated by the *arrows*.

As described above, chimaerins have a RacGAP domain and therefore accelerate the hydrolysis of GTP from Rac, leading to its inactivation. Given the high-affinity binding of phorbol esters for chimaerins and their effects on chimaerin translocation, a question arises: can phorbol esters regulate chimaerin GAP activity? This hypothesis was initially explored for α1-chimaerin using Rac-GTP hydrolysis assays, which revealed low albeit significant increases in Rac-GAP activity by PMA (Ahmed et al., 1993). Similar experiments performed with β2-chimaerin show no significant changes in Rac-GAP activity in the presence of PMA (M. J. Caloca, H. Wang, and M. G. Kazanietz, in preparation). On the other hand, acidic phospholipids such as PS or PA markedly increase chimaerin GAP activity (Ahmed et al., 1993; Caloca et al., 2001). PMA promotes the association of β2-chimaerin with RacV12 (an activated form of Rac) in COS-1 cells, as judged by coprecipitation assays (Caloca et al., 2001). Taken together, these results support a "positional" model in which phorbol esters (and probably DAG) primarily redistribute β2-chimaerin to membranes where it binds Rac, and allosteric activation is triggered by membrane phospholipids. It remains to be explored how redistribution of chimaerins to the perinuclear region relates to Rac signaling. Importantly, a large pool of Rac in its inactive, GDP-bound form is located in the perinuclear region (Kraynov et al., 2000). Therefore, it is tempting to speculate that β2-chimaerin and/or other chimaerin isoforms also play a role in the maintenance of the perinuclear Rac in an inactive state before this GTPase moves to the plasma membrane (Fig. 4).

There is strong experimental evidence that chimaerins inhibit Rac-mediated effects (Table 1). Among other functions, Rac is involved in actin cytoskeleton reorganization, adhesion, migration, and cell cycle control (Coso et al., 1995; Ridley, 1996; Kjoller and Hall, 1999; Schmitz et al., 2000). Interestingly, ectopic expression of α1-chimaerin alters cytoskeletal and adhesive properties of NIH 3T3 fibroblasts. The assembly of integrin receptors, the organization of actin stress fibers and the formation of focal adhesions is also impaired (Herrera and Shivers, 1994). Expression of the α1-chimaerin GAP domain in leukocytes inhibits cytoskeletal responses to FMLP and CSF-1, and blocks phagocytosis, as also observed with a dominant negative (N17) Rac mutant (Cox et al., 1997). α2-Chimaerin is involved in neuritogenesis, and a role for the SH2 domain in α2-chimaerin has been proposed, suggesting that interaction with phosphotyrosine proteins yet to be identified may be critical (Hall et al., 2001). We have preliminary evidence that overexpression of β2-chimaerin impairs EGF signaling and cell proliferation, and it inhibits the metastatic potential of breast cancer cells (Lorenzano-Menna et al., submitted; M. J. Caloca, H. Wang, and M. G. Kazanietz, in preparation). Interestingly, a marked reduction in β2-chimaerin expression was observed in high-grade astrocytomas, suggesting a dysregulation of Rac signaling in tumor progression (Yuan et al., 1995).

Munc13 Isozymes: Phorbol Ester Activation and Exocytosis. With the exception of a Munc13-2 splice vari-

ant, which is ubiquitously expressed, Munc13 proteins are mainly expressed in brain (Augustin et al., 1999; Koch et al., 2000). Evidence for phorbol ester-induced translocation of Munc13 isozymes has been reported in human embryonic kidney 293 cells transiently transfected with GFP-fused Munc13 constructs. These experiments show that all three Munc13 isoforms translocate to the plasma membrane in response to PMA. The involvement of the C1 domain was confirmed in experiments showing that a mutant insensitive to phorbol esters does not redistribute. On the other hand, a truncated mutant of Munc13-1 comprising only its C1 domain translocated in response to PMA (Betz et al., 1998). Despite some evidence of translocation of Munc13 to the Golgi in response to phorbol esters, the biological relevance of this redistribution has yet to be determined (Song et al., 1999).

Munc13 isoforms act as scaffolding proteins that interact with elements of the exocytotic machinery, such as syntaxin, Doc2, RIM1, and spectrin (Betz et al., 1997, 2001; Orita et al., 1997; Sakaguchi et al., 1998; Duncan et al., 1999), and therefore play an essential role in exocytosis (Table 1). Munc13-1 acts as a factor that transfers unprimed vesicles to a pool of release-competent, primed vesicles. Phorbol esters promote a transient interaction of Munc13-1 with the calcium-binding protein DOC2. This association was independent of PKC activation and required an intact Munc13 C1 domain (Orita et al., 1997; Duncan et al., 1999). This event may be important in the regulation of vesicular trafficking and is probably a key step in phorbol ester-dependent enhancement of exocytosis from presynaptic terminals. The importance of phorbol ester-regulated exocytosis via Munc13 was confirmed in experiments using microinjection of Munc13-1 mRNA into *Xenopus laevis* embryos. The gain-of-function effect of Munc13-1 occurs only when its C1 domain is intact (Betz et al., 1998).

RasGRPs: Phorbol Ester Activation of Ras Independent of PKC. The activation of the Ras cascade by phorbol esters has been extensively investigated in the last decade. Multiple points of cross talk between the PKC and Ras pathways, both upstream and downstream of Ras, have been reported (Cai et al., 1997; El-Shemerly et al., 1997; Marais et al., 1998; Schonwasser et al., 1998). The discovery of RasGRP1, a nucleotide exchange factor for Ras with transforming potential, uncovered a novel link between receptor-mediated stimulation of DAG signaling and Ras activation. It is well established that the C1 domain in RasGRP1 is critical for its activation in cellular models. Binding of PMA to the RasGRP1 C1 domain promotes its translocation from the cytosol to membrane fractions. In NIH 3T3 cells stably expressing RasGRP1, serum or PMA translocates RasGRP1 to cellular structures around the nucleus and to the cell periphery (Ebinu et al., 1998; Tognon et al., 1998). A recent interesting study by Lorenzo et al. (2001) showed a biphasic translocation of RasGRP3: at lower PMA concentrations, it translocates predominantly to the plasma membrane, and at higher concentrations, a perinuclear and nuclear membrane distribution was observed. Colocalization with a Golgi marker was detected in the perinucleus.

RasGRP1 may serve as a direct link between receptors coupled to DAG generation and Ras activation at the plasma membrane. A schematic model of RasGRP1 regulation is depicted in Fig. 5. Expression of RasGRP1 increases the GTP loading of Ras, an effect that is further increased by PMA (Ebinu et al., 1998). Although the involvement of phorbol ester-responsive PKCs has not been ruled out, similar experiments using RasGRP3 showed that the increase in Ras-GTP loading by PMA cannot be blocked by a PKC inhibitor (Lorenzo et al., 2001). It seems that recruitment to the plasma membrane is sufficient to activate RasGRPs, as judged by the ability of a prenylated form of RasGRP1 to

TABLE 1
Cellular responses mediated by novel phorbol ester receptors

Phorbol Ester Receptors and Cellular Responses	Reference
α1-Chimaerin	
Inhibition of adhesion and regulation of cytoskeleton in fibroblasts	Herrera and Shivers (1994)
Control of lamellipodia/filopodia formation in neuroblastoma cells	Kozma et al. (1996)
Inhibition of membrane ruffling and phagocytosis in leukocytes	Cox et al. (1997)
Regulation of Golgi stability during interphase	Alonso et al. (1998)
α2-Chimaerin	
Inhibition of neuritogenesis	Hall et al. (2001)
β1-Chimaerin	
Potential role in acrosomal assembly and spermatogenesis	Leung et al. (1993)
β2-Chimaerin	
Control of progression in the development of astrocytoma	Yuan et al. (1995)
Differentiation of cerebellar granule cells	Leung et al. (1994)
Munc13 isozymes	
Neurotransmitter release	Betz et al. (1998)
Regulation of apoptosis in renal cells	Song et al. (1999)
Priming factor for vesicles in bovine chromaffin cells	Ashery et al. (2000)
Regulation of cerebellar synaptic transmission and motor learning	Augustin et al. (2001)
RasGRP1	
Transformation in fibroblasts and activation of ERK pathway	Ebinu et al. (1998); Tognon et al. (1998)
Thymocyte differentiation and TCR signaling	Dower et al. (2000); Ebinu et al. (2000)
RasGRP2/CalDAG-GEFI	
Control of proliferation and transformation in fibroblasts	Clyde-Smith et al. (2000); Dupuy et al. (2001)
Control of proliferation, adhesion and transformation in myeloblasts	Dupuy et al. (2001)
Coupling of M1 muscarinic acetylcholine receptors to ERK1/2	Guo et al. (2001)
RasGRP3	
Neuronal differentiation of PC12 cells	Yamashita et al. (2000)
Activation of ERK pathway in HEK293 cells	Lorenzo et al. (2001)

activate the Ras-dependent mitogen-activated protein kinase –ERK cascade (Tognon et al., 1998). A mutated RasGRP1 lacking the C1 domain failed to activate the ERK cascade and lost its characteristic transforming potential. Thus, the phorbol ester/DAG binding site has a dominant role in RasGRP1 activation. Further support for a link between DAG signaling and RasGRP has been recently provided in a study showing a direct association of RasGRP1 with DAG kinases (Topham and Prescott, 2001).

Unlike RasGRP1 and RasGRP3, the regulation of RasGRP2 by phorbol esters or DAG remains undefined. RasGRP2 has dual Ras/Rap1 GEF activity and is localized to the plasma membrane by post-translation modifications (palmitoylation and myristoylation). Its spliced variant CalDAG-GEFI, on the other hand, lacks the N-terminal consensus sequence for lipid modification and is confined to the cytosol. Despite the presence of a C1 domain, RasGRP2 fails to redistribute after phorbol ester treatment. Nevertheless, a substantial proportion of CalDAG-GEFI translocates to particulate fractions in cells treated with PMA for at least 15 min (Clyde-Smith et al., 2000). Although PMA enhances the Rap1GEF and RasGEF activities of RasGRP2 variants in COS cells (Clyde-Smith et al., 2000), evidence for a direct phorbol ester interaction using binding assays is still needed for RasGRP2.

It is conceivable that RasGRPs have the potential to contribute to the mitogenic and tumor promoting effects of the phorbol esters. In addition to its transforming potential in fibroblast models, RasGRP1 regulates thymocyte differentiation and T-cell activation. Overexpression of RasGRP1 in T cells enhances TCR-Ras-ERK signaling in response to calcium/PMA. In addition, RasGRP1 is differentially associated with membranes after TCR stimulation (Ebinu et al., 2000). Recent experiments illustrated that RasGRP1-null mutant mice have a significant reduction in the number of mature thymocytes. Remarkably, thymocytes from RasGRP1-deficient mice have a defective proliferative response and an impaired activation of the Ras-ERK cascade in response to PMA or anti-CD3 (Dower et al., 2000). Thus, RasGRP1 provides a nonredundant link between TCR ligation and activation of Ras signaling in thymocytes (Table 1).

Pharmacological Considerations: Should We Rethink Our View of Phorbol Esters as Selective PKC Activators?

The discovery of chimaerins, and more recently additional novel phorbol ester receptors, strongly supports the concept that a high degree of complexity exists in the pathways downstream of DAG generation. Many effects of phorbol esters that have been initially attributed to PKC isozymes may probably involve other targets and therefore require reevaluation. A similar concept applies to PKC inhibitors that target the phorbol ester binding site, such as calphostin C. It is clear now that this so-called "selective" PKC inhibitor blocks [^{3}H]PDBu binding not only to PKC isozymes but also to chimaerins, RasGRPs, and Unc-13 (Areces et al., 1994; Kazanietz et al., 1995b; Lorenzo et al., 2000). Thus, the use of calphostin C can lead to misleading conclusions, and great care should be taken in the interpretation of results. In light of the similar sensitivity of C1 domains to the archetypical phorbol esters and DAG, new strategies should be developed to achieve selective regulation of each pathway. The discrimination of targets by the analog thymeleatoxin is a good example of how subtle differences in ligand recognition exist between different C1 domains, and a careful examination of these structural variables through modeling studies is probably the best approach for the rational design of C1 domain ligands.

An important emerging concept is that differential activation of phorbol ester receptors can be achieved by selective intracellular targeting. Proof-of-principle has been established for PKC isozymes using selective peptides targeted to protein-protein interaction sites (Csukai and Mochly-Rosen, 1999). Interesting studies by Wang et al. (1999, 2000) have recently revealed that selective translocation of PKC isozymes in cellular models can be achieved using different classes of analogs (such as bryostatin 1 or the novel DAG lactones) or by varying the ligand lipophilicity. Furthermore, we have recently demonstrated the selective activation of PKCα in prostate cancer cells by a rationally designed DAG analog (a DAG lactone). Despite its similar potency for PKCα and PKCδ in binding and kinase assays, this compound translocates each PKC to different intracellular compartments (Garcia-Bermejo et al., 2002). An important lesson from these studies is that marked discrepancies exist between in vitro and cellular effects of C1 domain-directed ligands and, more importantly, that in vitro pharmacological screenings may underestimate the selectivity observed in cellular assays. This novel pharmacological principle may prove to be useful in the design of selective analogs for each phorbol ester receptor class and in this way help to dissect DAG-mediated pathways as well as to elucidate the cellular functions of the novel phorbol ester receptors. It has been shown recently that DAG kinase γ binds [^{3}H]PDBu with high affinity through its C1A domain (Shindo et al., 2001). This is the first evidence that a DAG kinase is a specific phorbol ester receptor.

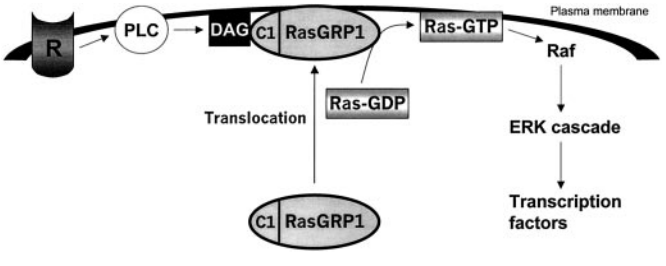

Fig. 5. Schematic of RasGRP1 activation. DAG binds to the C1 domain of RasGRP1 in the plasma membrane. Consequently, RasGRP1 promotes GDP/GTP exchange on Ras, leading to the activation of the downstream Raf-ERK cascade.

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Correction to "Novel "Nonkinase" Phorbol Ester Receptors: The C1 Domain Connection"

Because of an error by the compositors, the above article [Kazanietz MG (2002) *Mol Pharmacol* **61:** 759–767] appeared in the April issue but was not correctly identified as a Minireview. The online version has been corrected.

We regret this error and apologize for any confusion or inconvenience it may have caused.