A Novel Diacylglycerol-lactone Shows Marked Selectivity in Vitro among C1 Domains of Protein Kinase C (PKC) Isoforms α and δ as Well as Selectivity for RasGRP Compared with PKCα*

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Although multiple natural products are potent ligands for the diacylglycerol binding C1 domain of protein kinase C (PKC), RasGRP, and related targets, the high conservation of C1 domains has impeded the development of selective ligands. We characterized here a diacylglycerol-lactone, 130C037, emerging from a combinatorial chemical synthetic strategy, which showed substantial selectivity. 130C037 gave shallow binding curves for PKC isoforms α, β, γ, δ, and ε, with apparent $K_i$ values ranging from 340 nM for PKCα to 29 nM for PKCe. When binding to isolated C1 domains of PKCα and -δ, 130C037 showed good affinity ($K_i = 1.78 \text{nM}$) only for δC1b, whereas phorbol 12,13-dibutyrate showed affinities within 10-fold for all. In LNCaP cells, 130C037 likewise selectively induced membrane translocation of δC1b. 130C037 bound intact RasGRP1 and RasGRP3 with $K_i$ values of 3.5 and 3.8 nm, respectively, reflecting 8- and 90-fold selectivity relative to PKCα and PKCe. By Western blot of Chinese hamster ovary cells, 130C037 selectively induced loss from the cytosol of RasGRP3 (ED50 = 286 nm), partial reduction of PKCe (ED50 > 10 µm), and no effect on PKCα. As determined by confocal microscopy in LNCaP cells, 130C037 caused rapid translocation of RasGRP3, limited slow translocation of PKCe, and no translocation of PKCα. Finally, 130C037 induced Erk phosphorylation in HEK-293 cells ectopically expressing RasGRP3 but not in control cells, whereas phorbol ester induced phosphorylation in both. The properties of 130C037 provide strong proof of principle for the feasibility of developing ligands with selectivity among C1 domain-containing therapeutic targets.

Diacylglycerol (DAG) is a lipid second messenger, produced through hydrolysis of phosphatidylinositol 4,5-bisphosphate following the activation of receptor-coupled phospholipase C or indirectly from phosphatidylcholine via phospholipase D (1). Most but not all effects of DAG reflect its interaction with proteins containing C1 domains, resulting in their activation and/or driving their membrane translocation. Reflecting the importance and diversity of its downstream effectors, DAG is involved in signal transduction of numerous physiological and pathological processes, including proliferation, differentiation, apoptosis, angiogenesis, and drug resistance (2). These functions have focused attention on C1 domain-containing proteins as molecular targets for cancer chemotherapy (3).

The interaction between DAG and its receptors is typically mediated by a DAG-responsive motif called a “C1 domain” (4). The highly conserved C1 domain (~50 amino acids) is a cysteine-rich zinc finger structure (5) that was first identified in protein kinase C (PKC) as the interaction site for DAG and the phorbol esters (6). The PKC family of serine/threonine protein kinases comprises the best studied mediators of DAG signaling. 8 of its 11 family members have DAG-responsive C1 domains: (i) the conventional PKCs (α, β, βII, and γ) and (ii) the novel PKCs (δ, ε, η, and θ). Both the classic and novel PKCs contain a C-terminal kinase domain and an N-terminal regulatory domain. The regulatory domain contains a pseudosubstrate domain, which occupies the catalytic site of the kinase domain and inhibits the kinase activity. Binding of DAG to the C1 domain completes a hydrophobic surface on the C1 domain, favoring its interaction with the membrane. The twin consequences are membrane translocation, controlling access to substrates, and a conformational change in PKC, removing the pseudosubstrate domain from the catalytic site, thereby activating the enzyme (7).

In addition to the PKC family, five other families of proteins (PKDs, RasGRPs, chimaerins, Munc13s, and DGKs) have been recognized with C1 domains responsive to DAG (7). The protein kinase D (PKD/PKCμ) family represents kinases superficially similar to PKC; however, the kinase domains are not homologous and show different selectivity. The PKDs lack the pseudosubstrate domain, show different spacing between their C1 domains, and contain a membrane-interacting pleckstrin homology domain (8). PKD is activated upon phosphorylation by PKC, with its C1 domains driving membrane localization. The Ras guanyl nucleotide-releasing protein family members (RasGRPs 1 to 4) function as guanine nucleotide exchange factors for Ras or Rap, leading to their activation (9, 10), as well as being subjected to PKC phosphorylation (11–13). The chima-
Novel Diacylglycerol-lactone with Marked Selectivity

rins are GTPase-activating proteins for Rac, leading to Rac inhibition. The Munc13 proteins are involved in the priming of vesicle fusion, and finally the DAG kinases function to abrogate DAG signaling, thus providing a negative feedback regulatory loop for the DAG signaling pathway (7).

Not only may DAG receptor families have complementary or antagonistic functions, but the same may be true within families. For example, PKCδ is growth-inhibitory in NIH3T3 cells, whereas PKCb and PKCd are growth-stimulatory (2, 3). Thus, complementary therapeutic strategies are to inhibit a specific PKC isoform or to stimulate an antagonistic isoform. For this latter approach, activators selective for different DAG receptors are needed.

A further level of complexity is that the conventional and novel PKCs, unlike the other classes of DAG receptors, have tandem C1 domains. It is becoming increasingly clear that different C1 domains may have different recognition properties and functions (14, 15). Therefore, compounds selective for individual C1 domains may have unique effects compared with less selective ligands (14).

Among the DAG receptors, particular attention has focused on PKC family members as therapeutic targets, because PKC isozymes play important roles in cell proliferation, tumor growth, and apoptosis (2). For example, elevated levels of PKCa, PKCγ, PKCe, and PKCζ have been found in central nervous system tumors (2). Overexpression of PKCa and PKCβ has been reported in breast cancer cells (2), and PKCβ is elevated in chemotherapy-resistant diffuse large B-cell lymphoma (16). On the other hand, there are data suggesting that some PKC isoforms, such as PKCδ, in many contexts promote apoptosis (17).

An underlying problem with the inhibition of PKC kinase activity as a therapeutic strategy is achievement of sufficient selectivity among serine/threonine specific protein kinases with homologous catalytic sites. A complementary strategy that we are pursuing has therefore been to design modulators targeted to the C1 domains. Dramatic variation in PKC behavior is provided through variability in the residues at the binding site in the C1 domains themselves, variation in other portions of the C1 domain that interact with the membranes, variations in other regions of the receptor that affect the membrane interactions (e.g., the pseudosubstrate region, the C2 domain, or the C terminus), differences in the lipid composition of different subcellular membranes, and differences among cells in membrane composition and in interacting proteins (3).

Multiple classes of high affinity ligands for PKCs have been described. These ligands include the diterpenes such as the phorbol esters, macrocyclic lactones such as the bryostatins, polyacetates such as aplysioatxin, or indole alkaloids such as teleocidin (3). Unfortunately, with the exception of the indole alkaloids, the complicated structures of these ligands have impeded their further development through medicinal chemistry. An alternative strategy, therefore, has been to use DAG derivatives in which the flexibility of the structure has been constrained to reduce the entropic loss due to binding (3). Using DAG-lactones, the Marquez group has been able to achieve potencies approaching those of phorbol esters (3).

The earlier natural products studies have highlighted the important role of the side chains in determining biological function. For example, 12-deoxyphorbol 13-tetradecanone is a tumor promoter, whereas 12-deoxyphorbol 13-acetate (prostratin) is an inhibitor of tumor promotion (18), and these compounds induce distinct patterns of localization of PKCδ in CHO cells (19). Similarly, unsaturated side chains promote inflammatory activity by phorbol esters but diminish their tumor promoting activity (20). Recently, the Marquez group has developed a combinatorial approach for exploring extensively the chemical space represented by these side chains (21). Here, we described our characterization of one of the early compounds emerging from this combinatorial chemistry approach. We report that the novel DAG-lactone derivative 130C037 displays marked selectivity among the recombinant C1a and C1b domains of PKCa and PKCδ. Likewise, 130C037 displays substantial selectivity for RasGRP relative to PKCα. These results provide strong encouragement for this drug discovery strategy directed at targets with C1 domains.

EXPERIMENTAL PROCEDURES

Materials—[20-3H]Phorbol 12,13-dibutyrate ([3H]PDBu) (20 Ci/mmol) was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO). PDBu and phorbol 12-myristate 13-acetate (PMA) were purchased from LC Laboratories (Woburn, MA). Phosphatidylserine was purchased from Avanti Polar Lipids (Alabaster, AL). Reagents for expression and purification of glutathione S-transferase (GST) fusion proteins were obtained from Pierce. Cell culture medium, reagents, and all of the DNA primers were obtained from Invitrogen. RasGRP3-GFP was generated as previously described (22). The mouse monoclonal anti-rabbit IgG was purchased from Roche Applied Science. The rabbit polyclonal anti-PKCa and anti-PKCe antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The anti-mouse IgG and anti-rabbit IgG were purchased from Bio-Rad.

Construction of GST- and GFP-fused C1 Domains of PKCa and PKCe—The C1a and C1b domains of PKCa and PKCe were generated by PCR using the Platinum® Pfx DNA polymerase (Invitrogen). The full-length cDNA clones of bovine PKCa and murine PKCe were used as the templates. The following oligonucleotides were used as the PCR primers to pull out the targeted C1 domains: (i) forward and reverse primers for cPKCa were 5′-AACGTCGACACGAGTGAA-3′ and 5′-CTTGTCTCCGGGTGCGGATAAG-3′; (ii) forward and reverse primers for cPKCe were 5′-CTTCTGCCTGGGTGCGGATAAG-3′ and 5′-GGTGTCCCAGGTATCCATGTCC-3′; (iii) forward and reverse primers for cPKCe were 5′-AACGACGGAAGATCCACTACA-3′ and 5′-GGTGTCCGGGTGCGGATAAG-3′; (iv) forward and reverse primers for cPKCδ were 5′-ACAGCCCAAGATCCACTACA-3′ and 5′-GGTGTCCGGGTGCGGATAAG-3′; (v) forward and reverse primers for cPKCδ were 5′-ACAGCCCAAGATCCACTACA-3′ and 5′-GGTGTCCGGGTGCGGATAAG-3′; (vi) forward and reverse primers for cPKCδ were 5′-ACAGCCCAAGATCCACTACA-3′ and 5′-GGTGTCCGGGTGCGGATAAG-3′; and (vii) forward and reverse primers for cPKCδ were 5′-ACAGCCCAAGATCCACTACA-3′ and 5′-GGTGTCCGGGTGCGGATAAG-3′.

The blunt-ended PCR products were ligated into a pCR®Blunt vector using the Zero Blunt® PCR cloning kit (Invitrogen). The pCR®Blunt vector was digested with EcoRI (New England Biolabs, Inc., Beverly, MA) to produce adhesives ends of the C1 fragment. This fragment was then ligated into the appropriate fluorescent protein-containing vectors (i.e., pEGFP-C1, pEGFP-C2, and pEGFP-GC3) (Clontech, Palo Alto, CA) and GST-containing vectors (i.e., pGEX-5X-1, pGEX-5X-2, and pGEX-5X-3) (Amersham Biosciences), using the EcoRI restriction sites with the extended reading frame. The DNA sequence of each construct was finally confirmed by sequencing analysis (DNA Minicore, Center for Cancer Research, NCI, National Institutes of Health).

Expression and Purification of the GST-tagged C1 Domains of PKCa and PKCe—The recombinant plasmids of individual C1 domains of PKCa and PKCe were transformed into BL-21-Gold (DE3) E. coli competent cells (Stratagene, La Jolla, CA). The expression of the GST fusion proteins was induced by the addition of 0.5 mM isopropyl-β-D-thiogalactopyranoside when the OD of the LB medium (Quality Biological, Inc., Gaithersburg, MD) reached 0.5–0.7. The bacteria were harvested after 4 h of induction at 37 °C. The expressed GST-tagged C1 protein was purified using a B-PER GST spin purification kit (Pierce). The purity of the protein was verified by SDS-PAGE and staining with Coomassie Blue. The protein concentration was measured using the Bio-Rad protein assay kit. The purified GST-C1 proteins were stored in 30% glycerol at ~70 °C.

[3H]PDBu Binding Assay—[3H]PDBu binding to the recombinant full-length PKC isoforms or the C1 domains of PKCs and PKCe was measured using the polyethylene glycol precipitation assay developed in our laboratory (25). Briefly, the assay mixture (250 μl) contained 50 mM Tris-HCl (pH 7.4), 100 μg/ml phosphatidylethanolamine, 4 mg/ml bovine immunoglobulin G, [3H]PDBu, 0.1 mM CaCl2 (for PKCa, -β, and -γ) and various concentrations of competing ligand. Incubation was carried out at 37 °C for 5 min (for full-length PKC isoforms) or 18 °C for 10 min (for C1 domains). Samples were chilled on ice for 7 min, 200 μl of 35% polyethylene glycol in 50 mM Tris-HCl (pH 7.4) was added. The samples were mixed and incubated on ice for an additional 10 min. The tubes were centrifuged and the supernatant was discarded. The pellets were dissolved in 0.2 ml of 0.5 M NaOH and 0.1 M EDTA and counted in a scintillation counter.
were centrifuged in a Beckman Allegra 21R centrifuge at 4 °C (12,200 rpm, 15 min). A 100-μl aliquot of the supernatant was removed for the determination of the free concentration of [3H]PDBu, and the pellet was carefully pelleted. The tip of the centrifuge tube containing the pellet was cut off and transferred to a scintillation vial for the determination of the total bound [3H]PDBu. Cytosolic (ICN, Costa Mesa, CA) was added both to the aliquot of the supernatant and to the pellet. Radioactivity was determined by scintillation counting. Specific binding was calculated as the difference between total and nonspecific binding. Standard Scatchard analysis was performed to determine the dissociation constants (Kd) of the individual C1 domains, and the inhibitory dissociation constants (Ki) were calculated using our standard method as described previously (23).

Expression and Imaging of the GFP-tagged Fluorescent Protein in Live Mammalian Cells—LNCaP cells (obtained from ATCC, Manassas, VA) were cultured at 37 °C in RPMI 1640 containing 10% fetal bovine serum, penicillin (50 units/ml), and streptomycin (0.05 mg/ml) in a 5% CO2 atmosphere. The plasmid DNA of GFP-fused individual C1 domains and RasGRP3 was subcloned into the modified pLenti6/V5-D-TOPO® vector by using the Lipofectamine 2000 (Invitrogen). CHO-K1 cells (obtained from ATCC, Manassas, VA) were seeded in 60-mm tissue culture plates and serum-starved overnight before treatment with a series of concentrations of 130C037 or PMA for 30 min at 37 °C. The cells were washed once with Dulbecco’s phosphate-buffered saline and then lysed with 0.2 ml of M-PER mammalian protein extraction reagent (Pierce). The lysates were collected and transferred to 1.5-ml centrifuge tubes. Cell debris was pelleted by centrifugation at 14,000 × g for 10 min. The supernatants were transferred to new tubes, and the protein concentration was measured using Bio-Rad protein assay reagent. The samples, each containing 50 μg of total protein, were separated by electrophoresis on 10% SDS-polyacrylamide gels, and the protein bands were transferred onto Immobilon-P membranes (Millipore Corp.). After the membranes were blocked with 1% phosphate-buffered saline containing 0.1% Tween 20 (Bio-Rad, Hercules, CA) and 5% nonfat milk for 1 h, the blots were probed with anti-phospho-44/42 mitogen-activated protein kinase monoclonal antibody (Cell Signaling Technology). Then the blots were stripped and reblotted with anti-RasGRP3 antibody (Cell Signaling Technology) and anti-V5 antibody (Invitrogen). The blots were then developed with the ECL system (Amersham Biosciences) and imaged on BioMax MAX films (Kodak).

RESULTS

Chemical Structure of 130C037 and 130C032 as Novel DAG-lactones—As described in detail elsewhere, we have developed a combinatorial chemical approach for the synthesis of DAG-lactones (21). As part of our further analysis of some of the initial compounds emerging from that program, two compounds showed atypical binding curves with PKCα and were therefore examined in detail. The structures of these compounds, 130C037 and 130C032, are illustrated in Fig. 1. Both compounds differ from most DAG-lactone or phorbol ester derivatives that have been described in the literature in that the sn-1 and sn-2 substituents possess polar groups (3).

130C037 and 130C032 Yielded Biphasic Binding Curves with PKC isoforms in Vitro—The binding of 130C037 and 130C032 to recombinant PKCα, PKCβ, PKCγ, PKCδ, and PKCe was determined in vitro by competition with [3H]PDBu (Fig. 2). Unlike typical competitive binding curves, these curves were shallow and multiphasic both for 130C037 (Fig. 2A) and 130C032 (Fig. 2B). In addition, particularly at higher ligand concentrations, they showed appreciable differences between PKC isoforms. This is most evident comparing the curves for the classic PKCs α, β, and γ and for the novel PKCs δ and ε at
higher concentrations of 130C037. Of these two compounds, 130C037 showed a greater range of apparent potencies. When we analyzed the binding curves using a single site model, the apparent $K_i$ values of 130C037 and 130C032 for the five PKC isoforms ranged from $\sim$30 to 350 nM (as shown in Table I). Because of the shallow nature of the binding curves, the calculated $K_i$ values should be regarded as approximations.

**Comparison of the Binding Affinities of 130C037 for Isolated C1a and C1b Domains of PKCα and PKCδ—**Since some ligands have been found to show selectivity among individual C1 domains (14, 15, 24), we measured the binding of 130C037 to the purified, individual C1a and C1b domains of PKCα and PKCδ, which we expressed in *E. coli* as GST fusion proteins. We found dramatic differences in the binding to the different C1 domains (Fig. 3 and Table II). 130C037 bound with high potency ($K_i = 1.78 \pm 0.51$ nM, mean ± S.E., $n = 3$ experiments) to the PKCδ C1b domain, whereas its affinity for the PKCδ C1a domain was 3 orders of magnitude less, with a $K_i$ of 2780 ± 900 nM (mean ± S.E., $n = 3$ experiments). This difference markedly contrasts with that for PDBu, which bound with high affinity to both C1 domains, although its affinity for the δC1a domain was slightly weaker (6.2-fold) than for δC1b. Thus, whereas 130C037 was modestly less potent than PDBu for δC1b (5.4-fold), it was 1360-fold less potent for δC1a.

130C037 bound with substantially weaker affinity to either C1 domain of PKCα than it did to the δC1b domain (Fig. 3B, Table II). Its affinity for αC1a was 610 nM; for αC1b no binding was detectable. PDBu, in contrast, bound to both αC1a and αC1b with high potency ($K_i = 0.40$ and 3.40 nM, respectively). Thus, 130C037 was 1530-fold less potent than PDBu at αC1a and was >2900 less potent at αC1b.

We conclude that 130C037 demonstrated marked selectivity between C1 domains, having high affinity only for the δC1b among the four domains examined. This selectivity contrasts with the very modest selectivity observed for PDBu, arguing against artifacts in our experiments that would simply render a C1 domain inactive for binding. This is the first synthetic DAG analogue to have been reported so far with such high selectivity (>1000-fold difference in $K_i$ values) for the individual C1 domains. An important note of caution is required, however. Comparison of the binding affinity values for the individual C1 domains of PKCα and PKCδ with those for the intact PKC isoforms did not provide good agreement (also see below). This is consistent with the expectation that the binding properties of the intact isoforms will depend on the full complement of structural features that determine the energetics of formation of the ternary/quaternary complex between PKC, ligand (at one or two sites), membrane, and calcium. Although evaluation of the binding characteristics of isolated C1 domains may be informative, ultimate evaluation will require assessment of the behavior of the intact protein, preferably in the context of the target cell.

**Translocation of Individual C1a and C1b Domains of PKCα and PKCδ by 130C037 in Single Live LNCaP Cells—**The C1 domain selectivity of 130C037 was further examined in live cells. We prepared fusion constructs between the C1a and C1b domains of PKCα and PKCδ and GFPs. The constructs were transfected into LNCaP cells, and the translocation of the overexpressed GFP-C1 was monitored by confocal microscopy as a function of time after the addition of 130C037. GFP-αC1a, GFP-αC1b, and GFP-δC1b were all almost evenly distributed inside the cell (Fig. 4, A and B, first column), whereas GFP-δC1a predominantly accumulated inside the nucleus (first column in panel 3 of Fig. 4A). After the application of 130C037 (20 μM), translocation was only detected in the case of GFP-δC1b (Fig. 4B), No apparent translocation was seen for GFP-δC1a or for GFP-αC1a and GFP-αC1b under the same experimental conditions (Fig. 4A). In contrast, PMA induced a robust translocation for all four C1 domains (last column of Fig. 4, A and B).

We conclude that the *in vitro* results for the binding of 130C037 to the C1 domains were mirrored *in vivo* in its ability to selectively induce translocation of the C1 domains to the membrane in intact, live LNCaP cells.

**Comparison of the Binding Potency of 130C037 and 130C032 to RasGRP1/3 Versus PKCα and PKCδ—**We extended our characterization of 130C037 and 130C032 from PKC family...
members to the RasGRP family of DAG/phorbol ester-responsive signaling proteins. Unlike the PKC isoforms, RasGRP family members contain only one single C1 domain near their C terminus. In contrast to their weak to moderate binding to the PKC family members, 130C037 and 130C032 bound to both RasGRP1 and RasGRP3 with high affinity (Fig. 5, A and B, respectively). For comparison, the dose-response curves for PKCα and PKCε are also shown on the same graphs. The $K_i$ values of 130C037 for RasGRP1 and RasGRP3 were $3.51 \pm 0.06$ nM (mean ± S.E., $n = 3$ experiments) and $3.80 \pm 0.10$ nM (mean ± S.E., $n = 4$ experiments) (Table III), respectively, reflecting ~90-fold more potent binding than to PKCα and

![Figure 3](https://example.com/fig3.png)

**Fig. 3.** Dose-response curves of 130C037 for inhibition of binding of $[^{3}H]$PDBu to individual C1 domains of PKCδ (A) and PKCe (B). Binding of $[^{3}H]$PDBu to the individual C1 domains of PKCδ (A) and PKCe (B) was measured in the presence of different concentrations of 130C037. Binding assays were carried out as described under “Experimental Procedures.” Results are from single, representative experiments. Each experiment was repeated at least two additional times with similar results.

| PDBu, $K_i^{a}$ | 130C037, $K_i^{b}$ | Ratio of 130C037/PDBu |
|-----------------|------------------|-------------------|
|_nm_ | _nm_ | |
| $\delta$C1a | 2.04 ± 0.24 | 2780 ± 900 | 1363 |
| $\delta$C1b | 0.33 ± 0.05 | 1.78 ± 0.51 | 5.4 |
| Ratio of $\delta$C1a/$\delta$C1b | 6.2 | 1562 |
| $\alpha$C1a | 0.40 ± 0.11 | 610 ± 170 | 1525 |
| $\alpha$C1b | 3.40 ± 0.12 | $\geq 10,000$ | NA |
| Ratio of $\alpha$C1b/$\alpha$C1a | 8.5 | NA |

$^a$ $K_i$ was measured using a Scatchard assay.

$^b$ $K_i$ was measured using a competitive binding assay against $[^{3}H]$PDBu.

$^c$ NA, not applicable.

![Figure 4](https://example.com/fig4.png)

**Fig. 4.** Translocation of individual GFP-C1 domains of PKCe and PKCδ by 130C037 in LNCaP cells. LNCaP cells overexpressing different GFP-tagged C1 domains were treated with 130C037 (20 μM). The dynamic translocation of the green fluorescent proteins was recorded as a function of time after the addition of compound using a Bio-Rad MRC 1024 laser-scanning confocal microscope. A, time-serial images showing the translocation of GFP-$\delta$C1a (first row), GFP-$\alpha$C1b (second row), and GFP-$\delta$C1a (third row) by 20 μM 130C037. B, time-serial images showing the translocation of GFP-$\alpha$C1b by 20 μM 130C037. Translocation induced by PMA (1 μM; 5-min incubation) was displayed in the last column of both A and B as a positive control of the membrane translocation of the GFP-C1 constructs. The time in each panel represents the period after the drug administration. Images are from single, representative experiments. Each experiment was repeated at least two additional times with similar results.
8-fold more potent binding than to PKCe. 130C032 behaved similarly but showed slightly less selectivity. Unlike the dose-response curves for binding of 130C037 and 130C032 to the PKCs, the dose-response curves for binding to RasGRP1/3 were monophasic, unlike the biphasic curves observed for 130C037. We therefore examined the binding affinity of 130C045 for the isolated C1a and C1b domains of PKC and PKCβ to probe the relationship between the patterns of binding of the individual C1 domains and of the intact PKC. Like 130C037, 130C045 showed different binding potencies for the C1a and C1b domains of both PKCβ (Fig. 6C) and PKC (Fig. 6D). The average \( K_i \) values indicated that 130C045 had about 800-fold higher binding potency for \( \delta \)C1b (\( K_i = 1.54 \pm 0.22 \text{ nM, mean } \pm \text{ S.E.}, n = 3 \text{ experiments} \)) than for \( \delta \)C1a (\( K_i = 1229 \pm 25 \text{ nM, mean } \pm \text{ S.E.}, n = 3 \text{ experiments} \)). This difference was similar to that of 130C037 for \( \delta \)C1b and \( \delta \)C1a. The difference between \( \alpha \)C1a and \( \alpha \)C1b was less, only about 50-fold between the two \( K_i \) values (81.6 ± 6.3 nM for \( \alpha \)C1a and 4240 ± 500 nM for \( \alpha \)C1b, mean ± S.E., \( n = 3 \text{ experiments} \)). Since 130C037 had no detectable binding affinity for the \( \delta \)C1b domain of PKCα, quantitative comparison of 130C045 with 130C037 for selectivity between the \( \alpha \)C1a and \( \alpha \)C1b domains was not possible. However, the weaker binding potency of 130C045 for \( \alpha \)C1b as compared with \( \alpha \)C1a agreed qualitatively with that of 130C037. 130C045 thus had C1 domain selectivity for PKCβ and PKCα similar to that of 130C037. This result argues against the apparent biphasic inhibition curves of 130C037 for PKCα and PKCβ resulting simply from selectivity between C1 domains.

**Comparison of 130C037 and Its Positional Isomer 130C045 for Binding to PKC and RasGRP—**Computer modeling suggests that DAG-lactones can bind in either of two distinct orientations to C1 domains, depending on whether the binding utilizes the carbonyl of the sn-1 ester or the carbonyl of the sn-2 lactone (3). A critical consequence of the binding orientation is that it determines which side chain of the DAG-lactone projects away from the C1 domain into the lipid bilayer, and it would be expected that the more negatively charged \( p \)-nitrophenyl group would be disfavored for insertion into the bilayer. We therefore examined compound 130C045, the positional isomer of 130C037 in which the nitro- and dimethylamino-groups were swapped (see the insets in Fig. 6A). We compared the binding potency of this positional isomer for PKCs and RasGRP3 (Fig. 6, A and B) as well as for the C1a and C1b domains of PKCβ and PKCα (Fig. 6, C and D). In most respects, 130C045 behaved similarly to 130C037. The average \( K_i \) value of 130C045 for PKCα was 215 ± 14 nM (mean ± S.E., \( n = 3 \text{ experiments} \)). This value was comparable with that of 130C037 (i.e., 343 ± 35 nM). For RasGRP3, 130C045 bound with a \( K_i \) of 7.98 ± 0.94 nM (mean ± S.E., \( n = 3 \text{ experiments} \)); it was thus about 2-fold less potent than 130C037. The above results demonstrate that the selectivity between RasGRP3 and PKCα of 130C037 does not depend particularly on the positional isomerism of its two side chains.

Although 130C045 resembled 130C037 in its selectivity between RasGRP3 and PKCα, its binding curves for PKCα were monophasic, unlike the biphasic curves observed for 130C037. We therefore examined the binding affinity of 130C045 for the isolated C1a and C1b domains of PKCα and PKCβ to probe the relationship between the patterns of binding of the individual C1 domains and of the intact PKC. Like 130C037, 130C045 showed different binding potencies for the C1a and C1b domains of both PKCβ (Fig. 6C) and PKC (Fig. 6D). The average \( K_i \) values indicated that 130C045 had about 800-fold higher binding potency for \( \delta \)C1b (\( K_i = 1.54 \pm 0.22 \text{ nM, mean } \pm \text{ S.E.}, n = 3 \text{ experiments} \)) than for \( \delta \)C1a (\( K_i = 1229 \pm 25 \text{ nM, mean } \pm \text{ S.E.}, n = 3 \text{ experiments} \)). This difference was similar to that of 130C037 for \( \delta \)C1b and \( \delta \)C1a. The difference between \( \alpha \)C1a and \( \alpha \)C1b was less, only about 50-fold between the two \( K_i \) values (81.6 ± 6.3 nM for \( \alpha \)C1a and 4240 ± 500 nM for \( \alpha \)C1b, mean ± S.E., \( n = 3 \text{ experiments} \)). Since 130C037 had no detectable binding affinity for the \( \delta \)C1b domain of PKCα, quantitative comparison of 130C045 with 130C037 for selectivity between the \( \alpha \)C1a and \( \alpha \)C1b domains was not possible. However, the weaker binding potency of 130C045 for \( \alpha \)C1b as compared with \( \alpha \)C1a agreed qualitatively with that of 130C037. 130C045 thus had C1 domain selectivity for PKCβ and PKCα similar to that of 130C037. This result argues against the apparent biphasic inhibition curves of 130C037 for PKCα and PKCβ resulting simply from selectivity between C1 domains.

**Western Blot Analysis Showed Preferential Translocation of RasGRP3 by 130C037 as Compared with PKCα and PKCε in CHO Cells—**The *in vitro* analysis on the binding potency of 130C037 for PKC and RasGRP was carried out in the presence of 100 µg/ml phosphatidylserine, and it is well recognized that PKC isoforms may show different affinities and different structure-activity relations in intact cells. Indeed, structure activity relations even depend on the specific cell types (25, 26). We therefore examined three measures of response to 130C037 in intact cells. First, we determined the dose response for translocation of RasGRP3, PKCα, and PKCε by 130C037 in CHO-K1 cells using Western blot analysis. Because of the unavailability of anti-RasGRP3 antibody, the translocation of RasGRP3 was determined by using the overexpressed RasGRP3-FGP with anti-GFP antibody. The response of the endogenous PKCα and PKCε was monitored using anti-PKCα and anti-PKCε antibodies. Under our experimental conditions, much of the examined...
proteins was already in the membrane fraction of the control samples; we therefore quantitated the reduction of the cytosolic fraction as an index of membrane translocation of the proteins. No change in cytosolic PKCa distribution was observed at any concentration of 130C037 over the range investigated (Fig. 7B). Partial loss of RasGRP3 and PKCe from the cytosol was observed (Fig. 7, A and C). However, the quantification of the bands in the cytosolic fractions demonstrated that 130C037 was much more potent for translocating RasGRP3 than it was for translocating PKC\(\alpha\) (Fig. 7D). The average ED\(_{50}\) for RasGRP3 from five independent experiments was 286 ± 12 nm (mean ± S.E.); the ED\(_{50}\) for PKCe was more than 10 \(\mu\)M. Only minor loss of RasGRP3 or of PKCe from the total fraction was seen, arguing against down-regulation or cell loss under these conditions of time and concentration accounting for the response. Although PKCa did not translocate in response to 130C037, it translocated in response to PMA (10 nm, 100 nm, 1 \(\mu\)M, and 10 \(\mu\)M) under these conditions (three experiments, data not shown), indicating that the lack of translocation of PKCa in response to 130C037 was not an artifact. We conclude that, as in vitro, in intact cells RasGRP3 was the most sensitive receptor for 130C037 when comparing with PKCs and PKCa. It is also clear from this analysis that 130C037 was much less potent in the intact cell system than in the in vitro assays. This reduction in apparent potency of DAG-lactones in cellular systems is typical (e.g. see Ref. 27).

Translocation of RasGRPs-GFP, PKC\(\alpha\)-GFP and PKCe-GFP in Response to 130C037 in Live LNCaP Cells—As a complementary approach to confirm the in vivo selectivity of 130C037 for RasGRP3, we investigated the dynamic translocation of GFP-tagged RasGRPs, PKC\(\alpha\), and PKCs in LNCaP cells, a prostate cancer cell line. The dynamic redistribution of RasGRPs-GFP, PKC\(\alpha\)-GFP, and PKCe-GFP in live LNCaP cells after the application of 130C037 was monitored using a Bio-Rad MRC 1024 confocal microscope. As a positive control, response to PMA was determined. RasGRPs-GFP translocated to internal membranes after the application of 130C037 (20 \(\mu\)M), similar to its response to PMA (Fig. 8A). PKC\(\alpha\)-GFP did not show any membrane translocation in response to 130C037 but showed a clear response to PMA (Fig. 8B). Unlike PKC\(\alpha\)-GFP, PKCe-GFP showed a slow, partial plasma membrane translocation in response to 130C037 (20 \(\mu\)M). This live cell imaging confirmed again, in a second cell type under different conditions, that in vivo 130C037 was most sensitive for RasGRPs, least active on PKC\(\alpha\), and of intermediate activity on PKCe.

Erk1/Erk2 Phosphorylation Induced by 130C037 but Not by PMA Was Dependent on RasGRPs—As a third measure of selectivity of 130C037 for RasGRPs in intact cells, we examined the functional response of Erk phosphorylation. Early studies of PMA-induced Erk activation indicated that stimulation of PKC could lead to activation of Raf in a manner that depends on basal levels of Ras activity but does not involve overt Ras activation (28). Although the mechanistic details of this process have not been fully elucidated, it may reflect a ubiquitous process, because PMA activates Erk in many cell types. In contrast, in select cell types, including lymphocytes, PMA activates RasGRPs family members. This process probably depends on direct recruitment of RasGRPs to membranes where it can catalyze conversion of Ras-GDP to Ras-GTP that contributes to Ras activation (28). Besides these two distinct mechanisms of PMA-induced Erk activation, there is growing evidence that PKC and RasGRPs signaling mechanisms can intersect. In particular, RasGRPs requires phosphorylation by PKC for full activation, which may be mediated by co-recruitment of these proteins to common membranes (11–13).

We compared the ability of 130C037 and PMA to induce

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**Fig. 6. Dose-response curves of 130C037 and 130C045 for binding to PKC\(\alpha\) (A) and RasGRPs (B) and the dose-response curves of 130C045 for binding to individual C1 domains of PKC\(\delta\) (C) and PKC\(\alpha\) (D). The inhibition of [\(^{3}H\)PDBu binding to the different receptors in the presence of 130C037 or 130C045 with different concentrations was determined as described under “Experimental Procedures.” Results are from single, representative experiments. Each experiment was repeated at least two additional times with similar results. The chemical structures of 130C037 and 130C045 are shown in the inset of A.**
Erk1/Erk2 phosphorylation in control HEK-293 cells and in HEK-293 cells stably overexpressing RasGRP3 (Fig. 9). PMA induced Erk phosphorylation in the control cells, reflecting the response mediated by the endogenous PKC (Fig. 9A, left half). This response was inhibited by treatment with the PKC inhibitor GF109203X (data not shown). In the HEK-293 cells heterologously expressing RasGRP3, PMA likewise induced Erk phosphorylation as expected, reflecting its combined effect on endogenous PKC and the heterologously expressed RasGRP3 (Fig. 9A, right half). In contrast to PMA, 130C037 up to a concentration of 40 μM was unable to induce Erk phosphorylation in the control HEK-293 cells (Fig. 9B, left half), whereas it was able to do so in the RasGRP3-overexpressing cells (Fig. 9B, right half). Controls for Erk protein confirmed equal load-
ing in all lanes, and controls for the V5 epitope confirmed equal levels of the RasGRP3 in the lanes with the RasGRP3-expressing cells. To eliminate an alternative explanation, that PKC was up-regulated in the RasGRP-overexpressing cells, we examined the protein level of different PKC isoforms in both control and RasGRP3-overexpressing cells by Western blot analysis. No difference was observed (data not shown). We conclude that 130C037 is selective for inducing functional responses through a RasGRP3-dependent pathway in this intact cell system.

DISCUSSION

The diacylglycerol signaling pathway has gained great attention as a central regulatory mechanism in cells, contributing to the control of cell proliferation, differentiation, and apoptosis. PKC, the major receptor for DAG and the tumor-promoting phorbol esters, has thus emerged as an attractive therapeutic target for cancer and a range of other conditions. Complementing the design of inhibitors targeted to the catalytic domain of PKC, we and others have pursued the design of ligands targeted to the C1 domains involved in PKC activation (3, 14). The rationale is that different PKC isoforms may produce functional antagonism, and activation of an antagonistic PKC may achieve the same result as inhibition of the complementary isoform. With the identification of other families of signaling molecules containing DAG-responsive C1 domains (e.g. RasGRP, PKD, or chimaerin), both the challenges for designing specificity and the opportunities have increased.

We are still in the early stages of exploring the full potential of constrained DAG derivatives as probes of the function of C1 domain containing receptors and as lead molecules in drug discovery. Extensive analysis has identified a potent DAG-lactone (130C037) that was selective in vitro for binding to RasGRP1/3 as compared with PKCα. This is the one of the first studies so far reporting a compound selective for RasGRP and the first example of a compound with such a degree of selectivity for a DAG receptor other than PKC. Previously, we have shown that the iridal NSC631939 bound to RasGRP with 5-fold selectivity relative to PKCα (32). We have shown in this study that the novel DAG-lactone 130C037 had a much stronger binding affinity for RasGRP1/3 \((K_i = 3.8 \pm 0.1 \text{ nM})\) and \(K_i = 3.51 \pm 0.06 \text{ nM}\) than for PKCα \((K_i = 343 \pm 35 \text{ nM})\). The Western blot analysis of RasGRP3 and PKCα translocation in CHO-K1 cells also demonstrated that under similar experimental conditions 130C037 could only translocate RasGRP3 and not PKCα to the membranes (Fig. 7, A and B). Although the novel PKC isoform ε could respond to 130C037, the potency of 130C037 to induce membrane translocation of RasGRP3 \((ED_{50} = 286 \text{ nM})\) was appreciably stronger than for PKCε \((ED_{50} > 10 \text{ μM})\) (Fig. 7, C and D). The live cell imaging confirmed the selectivity of 130C037 in inducing RasGRP3-GFP translocation but not that of PKCα-GFP (Fig. 8, A and B). Under these conditions, PKCe-GFP showed weak plasma membrane translocation (Fig. 8C), which was consistent with the Western blot result. Finally, we have demonstrated that 130C037 could only induce Erk phosphorylation in HEK-293 cells exogenously expressing RasGRP3 (Fig. 9), whereas PMA could also induce its phosphorylation subsequent to the activation of the endogenous PKC in the cells.

The identification of 130C037 provides strong proof of principle for the potential of our combinatorial chemistry strategy and for the feasibility of designing compounds selective for members of the families of signaling molecules with DAG-responsive C1 domains. On the other hand, 130C037 has its limitations. Its activity in intact cellular systems is at the level of \(10^{-7} \text{ μM}\) in contrast to an in vitro potency of \(10^{-9} \text{ μM}\). It is somewhat less selective relative to PKCε than it is relative to PKCα or PKCδ. Finally, it appears to have a problem with solubility at micromolar concentrations, which we think contributes to the shallowness of the dose-response curves at the higher concentrations.

**FIG. 9.** Comparison of the induction of phosphorylation of Erk1/Erk2 in response to 130C037 and PMA in HEK-293 cells and in HEK-293 cells overexpressing RasGRP3. Serum-starved HEK-293 cells and HEK-293 cells overexpressing human RasGRP3 were incubated with the indicated concentrations of PMA (A) or 130C037 (B) for 30 min at 37 °C. The cells were washed with Dulbecco’s phosphate-buffered saline and then lysed. The cell lysates were separated by electrophoresis on 10% SDS-polyacrylamide gels and subjected to Western blotting with anti-phospho-Erk antibody. Equal loading of all lanes was confirmed by blotting with anti-Erk antibody, and for the RasGRP3-overexpressing cells, with antibody directed against the V5 epitope tag incorporated into the RasGRP3 construct.
Besides the selectivity of 130C037 for RasGRP versus PKCa and to a lesser degree PKCe, our study emphasizes that the behavior of C1 domains is strongly modulated by the context in which they are found in the intact proteins. As we have shown in the in vitro binding assays, the binding affinities of 130C037 for full-length PKCδ (or PKCa) and its isolated C1a and C1b domains clearly did not match. For example, the $K_r$ value of 130C037 for PKCδ was $91 \pm 16$ nM, but for $\delta$C1b it was $1.78 \pm 0.51$ nM. For $\delta$C1a, the $K_r$ was $2780 \pm 90$ nM. For PKCa, the $K_r$ of 130C037 (343 $\pm 35$ nM) was 4-fold weaker than that for PKCδ (91 $\pm 16$ nM), but its affinity for the better binding C1 domain, $\alpha$C1a (610 $\pm 170$ nM), was 350-fold weaker than that for $\delta$C1b (1.78 $\pm 0.51$ nM). Although the analysis of structure activity relations for the binding of ligands to isolated C1 domains, as so elegantly done by Irie et al. (14, 24, 33), should continue to be informative, particularly for identifying positive interactions, great caution should be exercised in interpreting negative results.

Presumably, a basis for the disparity between the binding to isolated C1 domains and to the intact receptors reflects the complexity of the interaction being measured, which is the formation of (at the very least) a ternary complex in which the ligand, the phospholipids, and the receptor all interact. As a simplified example, the addition of extra positively charged residues at the end of the C1 domain, in a position where they will not affect the direct interaction of ligand with the binding cleft but where they can affect charged interactions with the negatively charged phospholipids, had a substantial effect on the measured binding affinity (33). In the intact PKC, moreover, multiple domains contribute to the membrane interaction, including the pseudosubstrate region, the C1 domains, and Ca$^{2+}$-C2 domain complex. Furthermore, as suggested by Oancea and Meyer (34), the C1 domains appear to be buried and require a conformational change of the enzyme to become accessible. This same conformational change may help drive the extraction of the pseudosubstrate domain from the catalytic site of the enzyme. All of these coupled changes in interactions and conformational effects will necessarily be incorporated into the global energy change reflected in the apparent binding affinity of the ligand (35).

In the case of the PKC and PKD families of receptors, a further source of divergence in the behavior between the individual C1 domains and the intact proteins is that they contain two C1 domains. The relative contributions of these domains to the intact receptor remain uncertain, reflecting different analytic methodologies as well as different receptors being analyzed, for which the actual contributions may be different. For example, initial studies (6, 36) showed that both C1 domains of PKCγ bound PDBu with high affinity, suggesting that C1a and C1b of PKCγ are functionally equivalent. In contrast, other investigations (15, 31) suggested that the C1 domains in PKCa and PKCδ played somewhat different roles, depending on the specific ligand. Our results from 130C037 binding support non-equivalent roles of the C1a and C1b domains of PKCa and PKCδ, at least in terms of binding affinity for the DAG ligand. The development of ligands with marked selectivity among C1 domains should assist in the evaluation of their roles in the functioning of the intact receptors.
