Published in final edited form as:

FEBS J. 2010 January ; 277(1): 233–243. doi:10.1111/j.1742-4658.2009.07477.x.

Membrane anchoring of diacylglycerol-lactones substituted with rigid hydrophobic acyl domains correlates with biological activities

Or Raifman¹, Sofiya Kolusheva¹, Maria J. Comin², Noemi Kedei³, Nancy E. Lewin³, Peter M. Blumberg³, Victor E. Marquez², and Raz Jelinek¹

¹Department of Chemistry, Ben Gurion University, Beer Sheva 84105, Israel

²Laboratory of Medicinal Chemistry, Center for Cancer Research, National Cancer Institute at Frederick, National Institutes of Health, Frederick, MD 21702, USA

³Laboratory of Cancer Biology and Genetics, Center for Cancer Research, National Cancer Institute, Bethesda, MD 20892, USA

Summary

Synthetic diacylglycerol lactones (DAG-lactones) are effective modulators of critical cellular signaling pathways, downstream of the lipophilic second messenger diacylglycerol, that activate a host of protein kinase C (PKC) isozymes as well as other non-kinase proteins that share with PKC similar C1 membrane-targeting domains. A fundamental determinant of the biological activity of these amphiphilic molecules is the nature of their interactions with cellular membranes. This study characterizes the membrane interactions and bilayer anchoring of a series of DAG-lactones in which the hydrophobic moiety is a “molecular rod”, namely a rigid 4-[2-(R-phenyl)ethynyl]benzoate moiety in the acyl position. Application of assays employing chromatic biomimetic vesicles and biophysical techniques reveals that the mode of membrane anchoring of the DAG-lactone derivatives was markedly affected by the presence of the hydrophobic diphenyl rod and by the size of the functional unit displayed at the terminus of the rod. Two primary mechanisms of interaction were observed: surface binding of the DAG-lactones at the lipid/water interface and deep insertion of the ligands into the alkyl core of the lipid bilayer. These membrane-insertion properties could explain the different patterns of PKC translocation from cytosol to membranes induced by the molecular-rod DAG-lactones. This investigation emphasizes that the side-residues of DAG-lactones, rather than simply conferring hydrophobicity, profoundly influence membrane interactions and in that fashion may further contribute to the diversity of biological actions of these synthetic biomimetic ligands.

Keywords

Diacylglycerol; DAG-lactones; PKC translocation; membranes; membrane translocation

Introduction

The lipophilic second messenger *sn*-1,2-diacylglycerol (DAG) is released *in situ* from membrane phosphatidylinositol 4,5-bisphosphate through the action of phospholipase C in response to the occupancy of a wide range of G-protein-coupled receptors and receptor tyrosine kinases [1]. As a second messenger, DAG mediates the action of numerous growth factors, hormones and cytokines by activating members of the protein kinase C (PKC) family of enzymes, as well as several other families of signaling proteins, *e.g.* RasGRPs and chimaerins, that share with PKC the C1 domain as a DAG recognition motif. Many of these

signaling pathways feature prominently in the development and properties of cancer cells [2,3] and, in consequence, PKC isozymes are being actively pursued as therapeutic targets for cancer [4]. The majority of C1 binding ligands that are utilized are structurally rigid and complex natural products, such as the prototypical phorbol esters and the bryostatins [5]. These compounds bind their C1 receptors with nanomolar binding affinities and are greater than 3 orders of magnitude more effective than the very flexible, natural DAG agonists. In order to overcome this affinity gap and generate structures that are simpler and easy to synthesize, the Marquez group proposed to overcome the entropic penalty associated with the flexible glycerol backbone by constructing cyclic esters of DAG with the embedded glycerol backbone in various rigid conformations. In a comprehensive review, they discussed the reasons for selecting the five-member ring lactones, which are generically described as DAG-lactones [6]. Many of these DAG-lactones possess affinities for PKC approaching those of the phorbol esters and display marked diversity in the patterns of biological response that they induced as a function of the chemical nature of the side chains [6–9]. The concept that has emerged from these studies is that different patterns of substitution on the conformationally-restricted DAG-lactone template can preferentially interact with PKC isozymes within particular membrane microenvironments, promoting phosphorylation of those substrates co-localized with the activated PKC.

Previous results obtained with DAG-lactones containing acyl chains with an ensemble of repetitive oligo(*p*-phenyleneethynylene) units that form a rigid rod showed that two units constitute the ideal length of the rod [10]. The synthesis of several DAG-lactones fulfilling this structural constraint has already yielded important insights into the mechanisms of self-assembly and lipid interactions at the water/air interface and the diverse effects of different lipids upon the organization and thermodynamic properties of the molecules [11]. Because the end-residue of the rod (**R**) was shown to interact with the inner layer of the membrane and to modulate its surrounding environment, we focused in the present study on a group of compounds in which the **R** terminus of the rigid rod was varied from the smallest possible, *viz.* a hydrogen atom, to the bulkier isopropyl (*i*-Pr) and tertbutyl (*t*-Bu) groups (Table 1). As before, we compared these compounds to a DAG-lactone with a flexible decanoic acid chain (compound **1**, Table 1). The experiments were designed to explore the roles of both the rod side-residue as well as the properties of the larger alkyl **R** units in modulating bilayer interactions.

Here, we investigate the interactions and association of DAG-lactones **1–4** with lipid vesicles and correlate the data with their binding affinities for PKC- α and their abilities to induce translocation of PKC to cellular membranes. These ligands are shown to induce different patterns and kinetics for translocation of PKC isoforms to membranes and this study aims to examine whether membrane association might account for the biological differences. Application of several biophysical techniques, including biomimetic chromatic vesicles [12–14], fluorescence quenching [15], fluorescence anisotropy [16], differential scanning calorimetry [DSC] [17], and cryo-transmission electron microscopy (cryo-TEM) [18] reveal significantly different modes of bilayer binding of the rod compounds depending on their structure and highlight the role of the **R** terminus in affecting membrane insertion and biological activity of the DAG-lactones. The results shed light upon the molecular parameters affecting PKC translocation to membranes by DAG-lactones.

Results

Membrane translocation

To analyze membrane interactions of the DAG-lactones, we first evaluated their cellular effects. Compounds **2–4** showed similar, high affinity for PKC- α , as did DAG-lactone **1** in *in vitro* assays in the presence of 100 μ g/ml phosphatidylserine (Table 1). To study the

behavior of these DAG-lactones in living cells, we first determined the pattern and kinetics of the translocation of overexpressed, GFP-tagged PKC- α and PKC- δ to the membranes of Chinese hamster ovary (CHO) cells following addition of the compounds (Figure 1). As reported earlier [10], DAG-lactone **1**, included in this study as a DAG-lactone derivative which exhibits a highly flexible side-residue, translocated both PKC- α and - δ almost instantaneously to the cellular membranes, within less than 2 minutes (Figure 1A). Furthermore, **1** induced PKC- δ translocation simultaneously to the plasma membrane and to the internal membranes [10]. The translocation to the cellular membranes of both PKC- α and - δ was transient, unlike that caused by phorbol 12-myristate 13-acetate (PMA, the standard derivative used to characterize responses of PKC to phorbol esters or other ligands targeted to the C1 domain), or by the DAG-lactones containing rigid rod side chains described previously [10].

Figure 1 shows that DAG-lactone **2** is more similar to DAG-lactone **1** and DAG-lactone **3** is more similar to DAG-lactone **4** for inducing PKC translocation to the membranes. Specifically, DAG-lactones **1** and **2**, unlike PMA, gave rise to almost simultaneous translocation of PKC- δ to the plasma membrane, to the nuclear membrane, and to other internal membranes overall exhibiting a patchy distribution (Figure 1A–B, top row). In contrast, **3** and **4**, similarly to PMA, translocated PKC- δ in a sequential manner, initially to the plasma membrane and only later to the nuclear membrane and other internal membranes (Figure 1C and D, top rows). Additionally, the PKC- α translocation induced by **3** and **4** appears to be somewhat *slower* than the corresponding process induced by **1** and **2** (Figure 1A–D, bottom rows).

Chromatic vesicle analysis

To elucidate the mechanistic basis for the differences in the translocation patterns of PKC induced by DAG-lactones **2–4** apparent in Figure 1 we applied several biophysical techniques to characterize the membrane interactions of the molecules. Figure 2 depicts the concentration dependence of the fluorescence chromatic response (%FCR, see Materials and Methods) following addition of DAG-lactones **1–4** to biomimetic dimyristoylphosphatidylcholine (DMPC) / cholesterol / polydiacetylene (PDA) vesicles (mole ratio 1:1:3). Lipid/PDA vesicle assays have been employed for analysis of lipid-bilayer interactions of membrane-associated biological and pharmaceutical molecules [13,20]. Lipid/PDA vesicles consist of lipid bilayer domains (serving as biomimetic membrane docking areas) interspersed with PDA patches which act as the chromatic reporting units [12–14]. The specific lipid composition employed here, comprising DMPC and cholesterol, was designed to approximate cell-membrane environments [21].

The divergent chromatic dose-response curves in Figure 2 point to differences in membrane association of the DAG-lactones **1–4**. Specifically, **2** produced the most *moderate* increase in fluorescence chromatic response when incubated with the vesicles while **4**, in contrast, gave rise to the *steepest* %FCR dose-response curve (Figure 2). The chromatic response curves of **1** and **3** appeared in between the curves of **2** and **4** and were closer to **2**.

Previous studies have correlated the steepness (i.e. slope) of the chromatic dose response curves of lipid/PDA vesicles with the degree of *bilayer insertion* of the tested compounds [13,20]. Generally, molecules that penetrate deep into the hydrophobic core of the lipid bilayer induce *lower* chromatic transformations of the lipid/PDA vesicles (i.e. more *moderate increase* for the chromatic dose-response curves). On the other hand, substances which exhibit significant interactions with the lipid *surface* (lipid/water interface) were found to induce relatively more pronounced chromatic response (*steeper* dose response curves) [22]. According to this description, **2** most likely inserts deeper into the vesicle

bilayers compared to the other DAG-lactones examined, while **4** yields more pronounced surface interactions, consequently inducing the steeper dose-response curve in Figure 2.

Biophysical analysis

To further probe the interactions of the DAG-lactone **1–4** with lipid bilayers, particularly the extent of their localization at the vesicle interface, we carried out fluorescence quenching experiments utilizing DMPC/cholesterol vesicles into which the fluorescence probe NBD-PE was incorporated [15] (Figure 3). The NBD dye is embedded close to the bilayer interface, thus providing a useful marker for surface interactions of membrane-active compounds. The experiments summarized in Figure 3 depict the modulation of the fluorescence quenching of NBD by water-dissolved sodium dithionite following pre-incubation of the vesicles with the DAG-lactones, providing a measure of membrane interactions of the compounds [15].

Figure 3 demonstrates that incubation of the NBD-PE/DMPC/cholesterol vesicles with the DAG-lactones studied yielded significant changes in the rate of dithionite-induced fluorescence quenching of the bilayer-embedded dye. Importantly, all the DAG-lactones examined yielded *lower* quenching rates compared with the control vesicles (which were not pre-incubated with any DAG-lactone prior to addition of sodium dithionite). This result suggests that the NBD dye became more “shielded” from the soluble dithionite quencher as a consequence of vesicle binding by the DAG-lactones.

Figure 3 further shows that **3** and **4** induced more moderate quenching of vesicle-embedded NBD compared to **1** and **2**. This result is ascribed to greater shielding of the fluorescence dye affected through membrane interactions of **3** and **4**, implying that these ligands are more localized at the vesicle surface compared to **1** and **2**. The differences in fluorescence quenching profiles between DAG-lactones **2** and **4**, in particular, echo the chromatic experiment depicted in Figure 3, which pointed to relatively deeper insertion of **2** into the lipid bilayer compared to the pronounced surface interaction of **4**.

To further probe the effects of the four DAG-lactones on the cooperative properties and molecular organization of the lipid bilayer we examined the vesicles using differential scanning calorimetry (DSC) (Figure 4 and Table 2). Figure 4 depicts the effect of pre-incubating DAG-lactone **2** with DMPC/cholesterol vesicles. The thermograms in Figure 4 demonstrate that interactions of **2** with the phospholipids affected the peak position (i.e. the temperature in which the thermal transition, T_m , occurred [23]), the width at half-height ($T_{1/2}$, reflecting the ordering of phospholipid molecules undergoing the phase transition [23]), and the peak area (corresponding to ΔH , the overall enthalpy change associated with the thermal transition [23]).

The experimental parameters derived from the DSC experiments of DMPC/cholesterol vesicles incubated with DAG-lactones **1–4** are shown in Table 2. All four DAG-lactones significantly altered the DSC spectral parameters, a consequence of the interaction of the compounds with the lipid bilayer. Both T_m (maximum of DSC spectra) and ΔH (enthalpy change calculated from the peak areas) significantly decreased as a consequence of incubation of the DMPC/cholesterol vesicles with the DAG-lactones. However, the DSC parameters in Table 2 indicate that the compounds separate into two groups. Specifically, **1** and **2** gave rise to *lower* T_m values compared to **3** and **4**. Even more pronounced were the changes in the ΔH values. While **1** and **2** reduced ΔH by 1000 cal/mole or less, **3** and **4** yielded a decrease in ΔH of more than 2000 cal/mole (Table 2). This disparity between the two clusters (**1** and **2** vs. **3** and **4**) is similar to the fluorescence quenching results (Figure 3) and likewise is probably attributable to two distinct mechanisms of membrane interactions by the DAG-lactones (see Discussion section, below).

To further elucidate the effects of the DAG-lactones upon the dynamics of lipid molecules and upon bilayer fluidity we measured the fluorescence anisotropy of trimethyl-ammonium-1,6-phenyl-1,3,5-hexatriene (TMA-DPH), a widely-used probe which exhibits sensitivity to the dynamics of its lipid environment [24]. The DPH dye embedded in the lipid vesicles is located within the headgroup region close to the lipid/water interface [25] and thus yields insight into the dynamic consequences of molecular interactions at the bilayer surface [25].

Similar to the results of the fluorescence quenching (Figure 3) and DSC analysis (Table 2), the fluorescence anisotropy data in Figure 5 highlight two groupings among the DAG-lactones examined. Incubation of the TMA-DPH/DMPC/cholesterol vesicles with **1** and **2** resulted in relatively small changes in the fluorescence anisotropy of the lipid-embedded dye (Figure 5), indicating that lipid interactions of these two DAG-lactones had little effect upon the bilayer fluidity. In contrast, **3** and **4** gave rise to a significant reduction of fluorescence anisotropy, pointing to greater lipid mobility around the DPH probe [16].

To visualize the effect of the DAG-lactones on lipid vesicles we carried out cryo-TEM experiments (Figure 6). The cryo-TEM images in Figure 6 reveal the pronounced morphological consequences of DAG-lactone interactions with the vesicles and highlight the different effects induced by the ligands. Prior to addition of the DAG-lactones, the DMPC/cholesterol vesicles exhibit circular shapes with relatively uniform sizes having diameters that are smaller than 100 nm (Figure 6A) [18]. However, following incubation with **2** (Figure 6B) or with **4** (Figure 6C) the cryo-TEM images point to dramatic structural effects. DAG-lactone **2** appears to have induced internalization of vesicles within each other, giving rise to “onion-shape” structures (Figure 6B). **4**, on the other hand, induced the formation of giant vesicular structures, each comprising a single vesicle embedded within another, which do not exhibit precise circular structures (Figure 6C). While we cannot speculate on the exact mechanisms leading to the distinct structural transformations induced by the two DAG-lactones examined, the cryo-TEM data in Figure 6 clearly distinguish between bilayer interactions of **2** and **4**, consistent with the chromatic and biophysical analyses discussed above.

Discussion

The DAG-lactones substituted with rigid rods studied here are effective modulators of PKC both *in vitro* and in intact cells; however, differences in the patterns of PKC translocation to membranes were apparent between **1** and **2**, on the one hand, and **3** and **4** on the other hand (Figure 1). The experimental data presented here offer a mechanistic explanation for these differences and point to membrane interaction and insertion of the DAG-lactones as an important factor modulating the biological properties of these synthetic ligands.

Previous analysis of DAG-lactone libraries clearly indicated that the nature of the hydrophobic substituents on the DAG-lactones had a major influence on the specificity of their biological activities [7]. The present study provides direct evidence that the DAG-lactones **1–4** exhibit two distinct modes of bilayer interactions, closely affected by the biphenyl rods, and particularly the properties of the **R** terminus. Moreover, the two modes of membrane binding might account for the apparent differences in PKC translocation patterns and kinetics.

The experiments presented here point to deep bilayer insertion of **1** and **2** compared to localization of **3** and **4** at the lipid bilayer surface (Figure 7). This interpretation is supported by the chromatic vesicle data (Figure 2, although that experiment did not unequivocally distinguished surface binding of **3**), and the fluorescence quenching analysis (Figure 3). The

DSC experiments (Figure 4 and Table 2) highlight the pronounced effects of the DAG-lactones upon the structure of the lipid bilayers and corroborate the two distinct membrane interaction mechanisms proposed in Figure 7. Specifically, deep insertion of **1** and **2** into the bilayer is expected to modulate the lipid organization and consequently results in the significant changes in the position and width of the thermal transition (Table 2). In comparison, the association of **3** and **4** at the lipid/water interface essentially leads to “pinning down” of the lipid molecules in direct contact with the surface-attached DAG-lactones. These lipids, as a result, do not participate in the phase transition, thereby significantly reducing the ΔH values extracted from the DSC thermograms (Table 2).

The fluorescence anisotropy analysis (Figure 5) yields additional insight into the effects of the DAG-lactones upon the dynamic characteristics of the lipid bilayer. Figure 5 shows that **1** and **2** hardly modulate the fluorescence anisotropy of the DPH dye, consistent with deeper penetration of these compounds into the lipid bilayer and, as a consequence, lesser disruption of the bilayer interface at which the fluorescence probe is localized. In contrast, the enhanced fluidity (i.e. lower fluorescence anisotropy) apparent following incubation of the vesicles with **3** and **4** most likely corresponds to the pronounced interactions of these DAG-lactones with the lipid headgroup region.

Together, the experimental data demonstrate that the *size* of the side-residue **R** (Table 1) is most likely the primary factor determining the extent of binding and insertion of the DAG-lactones into the lipid bilayer. Paradoxically, although they increase the hydrophobicity of the molecule, bulky residues such as *i*-Pr (**3**) and *t*-Bu (**4**) appear to minimize penetration of the rods into the phospholipid bilayer, most likely resulting in their accumulation at the interface between the acyl chains and headgroup region of the lipids. On the other hand, when hydrogen is displayed at the rod terminus (DAG-lactone **2**), the ligand is capable of deep insertion into the more hydrophobic alkyl core of the bilayer. Our analysis also indicates that the *flexibility* of the DAG-lactone side-residue additionally contributes to bilayer insertion; **1**, which does not display the rigid diphenyl side-chain but rather a saturated alkyl side-chain of similar length, seems to penetrate deep into the lipid bilayer.

The two modes of DAG-lactone/membrane interactions might explain the differences in the patterns and kinetics of PKC translocation from cytosol to membranes observed for these ligands. The apparent insertion of **1** and **2** into the lipid bilayer most likely leads to anchoring of the molecules within the membrane and more effective binding between the DAG moiety and PKC, accounting for the rapid translocation of PKC- α and simultaneous translocation of PKC- δ onto all cellular membranes induced by these two compounds. In contrast, the interfacial bilayer adsorption of **3** and **4** probably disrupts the presentation of the DAG units, thereby slowing the translocation of both isoforms of PKC (apparent in Figure 1). This interpretation might also explain the lower translocation of PKC- δ to the internal membranes, which might be more sensitive to DAG-lactone orientation within the lipid bilayer.

Different patterns of interaction of synthetic DAG-lactone ligands with membranes afford both opportunities and challenges in drug design. We have previously described how the pattern of substitution can contribute to the orientation, either *sn*-1 or *sn*-2, of the insertion of the DAG-lactone into the binding cleft of the C1 domain [9]. The nature of *membrane insertion* of the ligand should likewise have great influence. Indeed, previous combinatorial analysis has illustrated the pronounced sensitivity and selectivity of biological response to the properties of the hydrophobic domain of the DAG lactone ligands [10]. Overall, this work indicates that the side-residues of DAG-lactones, rather than simply conferring hydrophobicity, affect membrane interactions of these synthetic ligands, thus directly modulating their biological functions.

Materials and Methods

Materials

Dimyristoylphosphatidylcholine (DMPC) was purchased from Avanti (Alabaster, AL). Sodium dithionite ($\text{Na}_2\text{O}_4\text{S}_2$) and cholesterol were purchased from Sigma. The diacetylenic monomer, 10, 12-tricosadiynoic acid, was purchased from Alfa Aesar (Karlsruhe, Germany) and purified by dissolving the powder in chloroform, filtering the resulting solution through a 0.45 μm Nylon filter (Whatman Inc.), and evaporation of the solvent. The fluorescent probes, N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)1,2-dihexadecanoyl-snglycero-3-phosphoethanolamine, triethylammonium salt (NBD-PE) and 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH) were purchased from Molecular Probes, Inc. (Eugene, OR, USA). Buffer solutions were passed through a 0.2 μm Nylon filter (Whatman Inc.) to remove impurities.

Synthesis

The syntheses of compounds **1** [10] and **2** [11] have been reported; similar synthesis procedures were employed for compounds **3** and **4**. The complete synthetic methodology and the full characterization of these compounds can be found in the Supporting Information.

Determination of binding of compounds to PKC—The binding affinity of ligands for murine PKC- α was determined by competition with $[20\text{-}^3\text{H}]$ phorbol 12,13-dibutyrate as described in detail elsewhere [26]. Briefly, binding of $[20\text{-}^3\text{H}]$ phorbol 12,13-dibutyrate to PKC- α was determined in the presence of the competing DAG-lactone. Assays were carried out for 5 min at 37 °C in the presence of 100 $\mu\text{g}/\text{ml}$ phosphatidylserine, 0.1 mM Ca^{++} , 50 mM Tris-Cl, pH 7.4, and 2 mg/ml IGG. The reaction mixture was then chilled to 4 °C and the protein- $[20\text{-}^3\text{H}]$ phorbol 12,13-dibutyrate complex was precipitated by addition of 35% polyethylene glycol. Samples were subjected to centrifugation, the supernatant removed, and radioactivity in pellet and supernatant was determined. Inhibition curves were determined using 7 concentrations of DAG-lactone, with triplicate determinations at each concentration in each experiment. The 50% inhibitory concentration of DAG-lactone was derived from the least squares fit of the data to a non-cooperative competition curve and the K_i was calculated from the 50% inhibitory value. Triplicate, independent experiments were performed for each DAG-lactone and K_i values presented represent the mean $\pm$ SEM of these triplicate experiments.

Measurement of translocation of GFP-tagged PKC isoforms α and δ to the plasma membrane and to internal membranes in response to ligand addition

—Chinese hamster ovary (CHO) cells were purchased from the American Type Culture Collection (Manassas, VA) and cultured in F12-K medium supplemented with 10% fetal bovine serum and antibiotics (penicillin at 50 units/mL and streptomycin at 0.05 mg/mL). CHO cells plated onto T delta dishes (Biotechs Inc., Butler, PA) were transfected with GFP-tagged PKC- α or PKC- δ using Lipofectamine reagent (Invitrogen, Carlsbad, CA). After 24 hours cells were visualized with a Zeiss LSM 510 confocal microscope (Carl Zeiss Inc., Thornwood, NY) with an Axiovert 100M inverted microscope operating with a 25 mW argon laser tuned to 488 nm. Cells were imaged with a 63×1.4 NA Zeiss Plan Apochromat oil immersion objective and with varying zooms (1.4–2). Time-lapse images were collected every 30 s before and after treatment with the indicated compounds (diluted in DMSO, final concentration 0.1%) using the Zeiss AIM software, in which the green emission was collected in a PMT with a LP 505 filter. All experiments are representative of at least 3 independent experiments.

Vesicle preparation

Preparation of vesicles containing DMPC, cholesterol, and the diacetylene monomer 10,12-tricosadiynoic acid (1:1:3 mole ratio) was carried out through dissolving the constituents in chloroform/ethanol (1:1) and drying together *in vacuo* to constant weight. This was followed by addition of deionized water to a final concentration of 1 mM and subsequently probe sonication at 70°C for 3 minutes. The vesicle solution was then cooled to room temperature and was kept at 4°C overnight prior to polymerization by irradiation at 254 nm for 0.5 min, resulting in an intense blue solution. Regular unilamellar vesicles composed of DMPC and cholesterol (1/1 mole ratio) were prepared through sonication of the aqueous lipid mixtures at room temperature for 10 min.

Multiwell fluorescence spectroscopy

Polydiacetylene (PDA) fluorescence was measured in 96 well microplates (Grainer) on a Fluscan Ascent (Thermo, Finland). All measurements were carried out at room temperature using 485-nm excitation and 555-nm emission using LP filters with normal slits. Acquisition of data was automatically performed every 40 seconds for 20 minutes, and the last measurement is presented. Samples comprised 30 μ L vesicle solution, DAG-lactone (1–20 μ L) followed by addition of 30 μ L 50 mM Tris-base buffer (pH 8.2).

A quantitative value for the extent of the blue-to-red color transitions within the PDA-labeled vesicles is given by the fluorescence colorimetric response (%FCR), which is defined as follows [20]:

$$\%FCR = [(F_I - F_0)/F_{100}] \times 100$$

where F_I is the fluorescence measurement of the vesicles after the addition of the compounds, F_0 is the fluorescence of the control sample (without addition of the compounds) and F_{100} is the fluorescence of a positive control sample (heated to produce the highest fluorescence emission of the red PDA phase).

Fluorescence quenching

NBD-PE was added to the DMPC/cholesterol vesicles at a molar ratio of 1:100 (probe : total phospholipids) and dried together *in vacuo* prior to sonication. Samples were prepared by mixing a selected amount of DAG-lactones with 30 μ L of the vesicles containing the fluorescent probe and 30 μ L of 50 mM Tris base buffer (pH 8.2), followed by addition of distilled water to a total volume of 1.5 mL. The quenching reaction was initiated by adding sodium dithionite, from a stock solution of 0.6 M in 50 mM Tris base buffer (pH 11), to give a final concentration of 1 mM. The decrease in fluorescence emission was recorded for 5 min at room temperature using 469 nm excitation and 530 nm emission on an Edinburgh Co. FL920 spectrofluorimeter (Edinburgh, Scotland, UK). The fluorescence decay curves were calculated as a percentage of the initial fluorescence measured before the addition of dithionite [15].

Fluorescence anisotropy

The fluorescence probe TMA-DPH was incorporated into the DMPC/cholesterol vesicles by adding the dye dissolved in THF (1 mM) to the vesicle solution and incubating for 30 min at room temperature. DAG-lactones were added to 30 μ L of the TMA-DPH/DMPC/cholesterol vesicles and 30 μ L buffer (pH 8.2) followed by addition of distilled water to a total volume of 1.5 mL. TMA-DPH fluorescence anisotropy was measured at 428 nm (excitation 360 nm) on an Edinburgh FL920 spectrofluorimeter. Anisotropy values (r) were automatically calculated by the spectrofluorimeter software using conventional methodology [16].

Differential scanning calorimetry (DSC)

DSC experiments were performed on a VP-DSC calorimeter (MicroCal, USA). Vesicle concentrations used in the experiments were 2 mM. Distilled water served as a blank. Heating scans were run at a rate of 1 °C min⁻¹. Data analysis was performed using Microcal Origin 6.0 software [17].

Cryogenic transmission electron microscopy (cryo-TEM)

Specimens studied via cryo-TEM were prepared in a similar manner as were the samples for the lipid/PDA vesicle analysis, described above. 4 µL sample solutions were deposited on perforated polymer films supported on a 300 mesh carbon-coated EM grid (copper, Ted Pella – lacey substrate). Ultrathin films (10–250 nm) were formed by removing most of the solution by blotting. The process was carried out in a vitrification system in which the temperature and relative humidity were controlled, using a Vitrobot (FEI) automatic system. Cryo-TEM images were recorded on a FEI Tecnai 12 G2 TWIN TEM equipped with a Gatan 626 cold stage [18].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

cryo-TEM	cryogenic transmission electron microscopy
DAG	diacylglycerol
DMPC	dimyristoylphosphatidylcholine
DSC	differential scanning calorimetry
%FCR	percentage fluorescence chromatic response
NBD-PE	N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)1,2-dihexadecanoyl- <i>sn</i> -glycero-3-phosphoethanolamine, triethylammonium salt
PDA	polydiacetylene
PKC	protein kinase C
TMA-DPH	1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene

Acknowledgments

We are grateful to Dr. L. Meshi and Dr. E. Nativ-Roth for help with the cryo-TEM experiments. This research was supported in part by the Intramural Research Program of the National Institutes of Health, Center for Cancer Research, National Cancer Institute.

References

1. Rhee SG. Regulation of phosphoinositide-specific phospholipase C. *Ann Rev Biochem.* 2001; 70:281–312. [PubMed: 11395409]
2. Griner EM, Kazanietz MG. Protein kinase C and other diacylglycerol effectors in cancer. *Nature Rev.* 2007; 7:281–294.
3. Nishizuka Y. Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. *Science.* 1992; 258:607–614. [PubMed: 1411571]
4. Mackay HJ, Twelves CJ. Targeting the protein kinase C family: are we there yet? *Nature Rev.* 2007; 7:554–562.

5. Choi SH, Hyman T, Blumberg PM. Differential effect of bryostatin 1 and phorbol 12-myristate 13-acetate on HOP-92 cell proliferation is mediated by down-regulation of protein kinase Cdelta. *Cancer Res.* 2006; 66:7261–7269. [PubMed: 16849575]
6. Marquez VE, Blumberg PM. Synthetic diacylglycerols (DAG) and DAG-lactones as activators of protein kinase C (PK-C). *Acc Chemical Res.* 2003; 36:434–443.
7. Duan D, Sigano DM, Kelley JA, Lai CC, Lewin NE, Kedei N, Peach ML, Lee J, Abeyweera TP, Rotenberg SA, et al. Conformationally constrained analogues of diacylglycerol. 29. Cells sort diacylglycerol-lactone chemical zip codes to produce diverse and selective biological activities. *J Med Chem.* 2008; 51:5198–5220. [PubMed: 18698758]
8. El Kazzouli SL, Lewin NE, Blumberg PM, Marquez VE. Conformationally Constrained Analogues of Diacylglycerol (DAG). 30. An Investigation of DAG-lactones Containing Heteroaryl Groups at the sn-2 Position Reveals Compounds with High Selectivity for RasGRPs. *J Med Chem.* 2008; 51:5371–5386. [PubMed: 18707088]
9. Pu Y, Perry NA, Yang D, Lewin NE, Kedei N, Braun DC, Choi SH, Blumberg PM, Garfield SH, Stone JC, et al. A novel diacylglycerol-lactone shows marked selectivity in vitro among C1 domains of protein kinase C (PKC) isoforms alpha and delta as well as selectivity for RasGRP compared with PKCalpha. *J Biol Chem.* 2005; 280:27329–27338. [PubMed: 15923197]
10. Malolanarasimhan K, Kedei N, Sigano DM, Kelley JA, Lai CC, Lewin NE, Surawski RJ, Pavlyukovets VA, Garfield SH, Wincovitch S, et al. Conformationally constrained analogues of diacylglycerol (DAG). 27. Modulation of membrane translocation of protein kinase C (PKC) isozymes alpha and delta by diacylglycerol lactones (DAG-lactones) containing rigid-rod acyl groups. *J Med Chem.* 2007; 50:962–978. [PubMed: 17284021]
11. Philosof-Mazor L, Volinsky R, Comin MJ, Lewin NE, Kedei N, Blumberg PM, Marquez VE, Jelinek R. Self-assembly and lipid interactions of diacylglycerol lactone derivatives studied at the air/water interface. *Langmuir.* 2008; 24:11043–11052. [PubMed: 18788772]
12. Okada S, Peng S, Spevak W, Charych D. Color and chromism of polydiacetylene vesicles. *Acc Chem Res.* 1998; 31:229–239.
13. Kolusheva S, Boyer L, Jelinek R. A colorimetric assay for rapid screening of antimicrobial peptides. *Nature Biotech.* 2000; 18:225–227.
14. Jelinek R, Kolusheva S. Polymerized lipid vesicles as colorimetric biosensors for biotechnological applications. *Biotech Adv.* 2001; 19:109–118.
15. McIntyre JC, Sleight RG. Fluorescence assay for phospholipid membrane asymmetry. *Biochemistry.* 1991; 30:11819–11827. [PubMed: 1751498]
16. Shinitzky M. Membrane fluidity in malignancy. Adversative and recuperative. *Biochim Biophysica Acta.* 1984; 738:251–261.
17. Gennis, BR. *Biomembranes: Molecular Structure and Function.* New York: Springer; 1989.
18. Talmon Y. Transmission electron microscopy of complex fluids: The state of the art. *Ber Bunsen-Ges Phys Chem Chem Phys.* 1996; 100:364–372.
19. Wildman S, Crippen G. Prediction of physicochemical parameters by atomic contributions. *J Chem Inf Comput Sci.* 1999; 39:868–873.
20. Kolusheva S, Shahal T, Jelinek R. Peptide-membrane interactions studied by a new phospholipid/polydiacetylene colorimetric vesicle assay. *Biochemistry.* 2000; 39:15851–15859. [PubMed: 11123911]
21. Spector AA, Yorek MA. Membrane lipid composition and cellular function. *J Lipid Res.* 1985; 26:1015–1035. [PubMed: 3906008]
22. Kolusheva S, Friedman J, Angel I, Jelinek R. Membrane interactions and metal ion effects on bilayer permeation of the lipophilic ion modulator DP-109. *Biochemistry.* 2005; 44:12077–12085. [PubMed: 16142905]
23. Mouritsen OG. Theoretical models of phospholipid phase transitions. *Chem Phys Lipids.* 1991; 57:179–194. [PubMed: 2054903]
24. Thomas B, Woolf BR. Structure, energetics, and dynamics of lipid-protein interactions: A molecular dynamics study of the gramicidin A channel in a DMPC bilayer. *Proteins.* 1996; 24:92–114. [PubMed: 8628736]

25. Lasch J. Interaction of detergents with lipid vesicles. *Biochimica et Biophysica Acta (BBA) - Rev Biomembr.* 1995; 1241:269–292.
26. Lewin NE, Blumberg PM. [^3H]Phorbol 12,13-dibutyrate binding assay for protein kinase C and related proteins. *Methods Molecular Biol.* 2003; 233:129–156.

Figure 1. PKC translocation

Confocal microscopy images of CHO cells overexpressing GFP-PKC- δ (top) and GFP-PKC- α (bottom), following treatment with: **A.** DAG-lactone **1**; **B.** DAG-lactone **2**; **C.** DAG-lactone **3**; **D.** DAG-lactone **4**. Final concentrations of all compounds were 10 μ M.

Figure 2. fluorescence dose-response curves of DMPC/cholesterol/PDA vesicles
The graph depicts the fluorescence emissions induced in the lipid/PDA vesicles following addition of DAG-lactones **1–4**.

Figure 3. Fluorescence quenching of NBD-PE embedded in DMPC/cholesterol vesicles
Fluorescence decay curves recorded following incubation of DAG-lactones **1–4** with NBD-PE/DMPC/cholesterol vesicles (1:50:50 mole ratio), followed by addition of sodium dithionite. **Control:** no DAG-lactone added.

Figure 4. DSC analysis

Effect of incubating DMPC/cholesterol vesicles with DAG-lactone **2** upon the DSC trace.

Control: no DAG-lactone added.

Figure 5. fluorescence anisotropy

Fluorescence anisotropy of DPH-TMA embedded in DMPC/cholesterol vesicles after addition of DAG-lactones **1–4**. **Control**: no compound added to vesicles.

Figure 6. Morphological effects of DAG-lactones probed by Cryo-TEM

A. Control DMPC/cholesterol vesicles (no DAG-lactones added); **B.** Vesicles incubated with **2**; **C.** Vesicles incubated with **4**. Bar corresponds to 100 nm.

Figure 7. Structural models

Schematic drawings depicting the proposed modes of association of the DAG-lactones with lipid bilayers. The overall length of the molecular-rod DAG-lactone is around 20 Å. **A.** deep bilayer insertion of **2**; **B.** surface binding of **4**;

Table 1

Structure and properties of the DAG-lactones.

1 2-4			
Compound	R	log P^a	K_i (nM) ^b
1	-	3.68	9.0 ± 1.2^c
2	H	3.26^d	6.6 ± 0.6^d
3	<i>i</i> -Pr	4.38	5.6 ± 1.1^c
4	<i>t</i> -Bu	4.56	5.3 ± 0.9^c

^aThe partition coefficient (octanol/water partition coefficient = log P) is a measure of the hydrophobic/hydrophilic balance of the molecule and is calculated by the atom-based program MOE SlogP [19]

^bThe K_i value measures the affinity of the ligand in terms of its ability to displace bound [20-³H]phorbol 12,13-dibutyrate (PDBU) from PKC- α . The lower the K_i , the more effective the ligand. Values representing the mean $\pm$ SEM of three independent experiments were determined as described in detail [11]

^cValues determined in the same set of experiments. For compound **1**, we had previously obtained and reported a value of 15.9 ± 1.1 nM [9].

^dValues from [11]

Table 2

Parameters extracted from the DSC thermograms.

DAG-lactone added	T_m	ΔH
Control (no addition)	23.1	3340
1	21.1	3000
2	20.6	2340
3	21.8	1380
4	21.8	1080

T_m : maximum of DSC spectrum (weighted average) ($^{\circ}\text{C}$); ΔH : enthalpy change (cal/mole).