CTP:phosphocholine cytidylyltransferase and protein kinase C recognize different physical features of membranes: differential responses to an oxidized phosphatidylcholine

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Abstract

Protein kinase C (PKC) and CTP:phosphocholine cytidylyltransferase (CT) are two examples of enzymes that are regulated by reversible binding to membranes, and this binding is influenced by membrane physical properties. CT activation by oxidized phosphatidylcholines was recently demonstrated and was linked to the acyl chain disordering effect of the oxidized species (Biochemistry 38, 15606). In this paper, we compare the responses of PKC and CT to an oxidized PC, and investigate the physical properties of lipid bilayers that modulate the activity of these enzymes. We show that 1-palmitoyl, 2-(11,15 dihydroxy) eicosatrienoyl PC (diOH-PAPC) caused less of an increase in the temperature of the lamellar to hexagonal II transition ($T_{H}$) of an unsaturated PE, compared to its parent, PAPC. Using a polarity-sensitive interfacial probe, we also found evidence to suggest that this oxidized PC increases interfacial packing pressure. We found that whereas diOH-PAPC activates CT, it inhibits PKC relative to the parent PAPC. The activities of both CT and PKC are known to increase in the presence of non-lamellar forming lipids. The greater activating effect of diOH-PAPC compared with PAPC, is consistent with a stimulation of the activity of CT by negative curvature strain. However, this is not the case with PKC, for which we suggest that surface packing pressure is of prime importance.

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1. Introduction

Oxidized lipids are generated during exposure of cell membranes to reactive oxygen species, for example in pathological states such as ischemia/reperfusion injury, and are also found in atherosclerotic lesions [1]. Some oxidized lipids have been shown to elicit a proliferative response from cells [2,3]. Polyunsaturated acyl chains are the primary targets for lipid oxidation. Various forms of both phospholipase A$_2$ and phospholipid glutathione peroxidases participate in the repair and elimination of the oxidatively damaged lipid species [4,5]. Nonetheless, oxidized phospholipids are inevitably present in cells because of the persistence of reactive forms of oxygen in the cellular environment. The membrane disordering effects of oxidized phospholipids have been clearly demonstrated by ESR, fluorescence and $^2$H-NMR methods [6–9]. Thus, it is pertinent to examine their effects on the function of membrane proteins. In this work, we investigate how the binding of amphitropic enzymes to membrane surfaces is influenced by physical changes promoted by oxidized lipids.

Amphitropic enzymes are a class of enzymes whose activities are modulated by the enzyme’s reversible trans-
location to membrane surfaces. This translocation can be regulated by the membrane lipid composition and by the membrane physical properties [10,11]. Two amphiprotic enzymes that respond to lipid compositional changes are protein kinase C (PKC; Refs. [12,13]) and CTP:phosphocholine cytidylyltransferase (CT; Ref. [14]). PKC is a family of Ser/Thr kinases involved in numerous signaling cascades [15]. CT catalyzes the regulatory step in the synthesis of phosphatidylcholine (PC), the predominant phospholipid of animal cell membranes and an important source of lipid signaling molecules [16]. Translocation of both enzymes to cell membranes is critical for their activation in vivo.

While both proteins bind interfacially, involving hydrophobic interactions [17,18], their membrane binding motifs are quite different. Classical PKCs bind membranes via the cooperation of C1 and C2 domains. The C1 domain binds a monomer of DAG, which generates a significant hydrophobic surface for membrane insertion [19,20], whereas the C2 domain binds 3 mol Ca\(^{2+}\) [21], one of which facilitates phobic surface for membrane insertion [19,20], whereas the C2 domain binds 3 mol Ca\(^{2+}\) [21], one of which facilitates its membrane binding [22]. Both domains have less well-defined binding sites for multiple anionic lipids. CT, on the other hand, has no pocket for a lipid monomer such as a C1 domain; instead, it contains a ~50 residue amphipathic α-helix which partitions into the membrane interfacial region with the helix axis parallel to the surface [23–26].

Membrane binding and activation of both PKC and CT are sensitive to changes in the physical properties of membranes that accompany compositional changes. Negative charge density is an important feature for both enzymes and is required for the electrostatic binding component [18,27,28]. CT and PKC also bind to bilayers enriched in so-called type II lipids, such as unsaturated PE, which induce negative curvature strain [10,29–32]. However, the mechanism for the activation by type II lipids of CT and PKC appears to be different.

In the case of CT, there is good evidence that the negative curvature strain induced in bilayers by these lipids is directly coupled with activation of the enzyme [31,32]. However, there are a number of indications that the mechanism is less directly related to negative curvature strain in the case of PKC. An initial indication of this is the finding that the activity of PKC can be assayed in Triton micelles [33], and membrane additives that promote negative monolayer intrinsic curvature activate PKC even in these positively curved structures [29]. This is not the case, however, for CT, whose activity is not enhanced by negative curvature agents when the enzyme is assayed in the presence of Triton micelles [28]. Further indication that the activity of PKC is not modulated directly by curvature strain is the observation that its activity is higher in the presence of lipids arranged in cubic phases than it is with lamellar phase lipid [34]. Since the cubic phase is formed at the expense of the lamellar phase in order to reduce curvature strain, the fact that PKC activity is higher in the cubic phase shows that the enhanced activity is not a consequence of increased curvature strain. Finally, PKC activation by a series of monounsaturated PEs with differing double bond position correlated poorly with the curvature strain associated with these PEs [35], but more closely with the surface polarity [36]. The latter was assessed by measuring the degree of penetration of a membrane-tethered fluorescent probe (DTMAC) into the bilayer, the depth of which is related to the interfacial polarity. A similar study with CT showed that the modulation of enzyme activity was directly related to curvature strain for these lipids [32].

The effects of oxidized lipids on PKC activity has been the subject of several reports, with conflicting results. Inhibition of PKC in vitro by oxidized phospholipids and oxidized arachidonic acid was observed in some cases [37], but activation by oxidized fatty acids was observed in other cases [38]. Oxidized DAGs increased PKC activity [39]. Increased lipid peroxidation in rat hepatocytes [40], glioma cells [41], and astrocytoma cells [42] was associated with PKC activation. None of these studies explored the effect of the physical alterations induced by membrane oxidation on PKC activity. A recent study showed that oxidized phospholipids activate CT in vitro, and the activation correlated with effects on acyl chain disordering [9]. The effects of an oxidized PC on both membrane order and CT activity were reversed by a saturated sphingomyelin [9].

We have examined the effects on membrane physical properties of a specific lipid oxidation product, 1-palmitoyl, 2-((11,15 dihydroxy) eicosatrienoyl) PC (diOH-PAPC). DiOH-PAPC is the major product of a lipoxygenase-catalyzed oxidation of 1-palmitoyl, 2-arachidonoyl PC (PAPC) [9]. DiOH-PAPC disorders acyl chains in bilayers [9]. Our objective in this study was to examine its effects on bilayer stability and lipid packing at the membrane interface. We compared the effects of this lipid with those of PAPC on the lamellar-to-hexagonal phase transition temperature (\(T_{\text{HI}}\)) of dielaidoyl PE (DEPE) using differential scanning calorimetry (DSC). We monitored the effects on interfacial polarity via changes in DTMAC fluorescence and quenching with doxyl PCs. The results suggest that the oxidized PC increases surface packing pressure. We also compared the effects of diOH-PAPC on CT and PKC activities, and found that the two enzymes respond in opposite ways to large unilamellar vesicles (LUVs) containing the oxidized PC. The results suggest that the oxidized lipid activates CT via effects on curvature strain, and inhibits PKC via effects on interfacial packing properties.

2. Experimental procedures

2.1. Materials

Egg PC, oleic acid, POPS, dielaidoyl PE (DEPE), PAPC, POPC, and spin-labeled 1-palmitoyl-2-stearoyl PC containing a doxyl spin label at position 5-, 7-, or 10 of the stearoyl chain were purchased from Avanti Polar Lipids.
DiOH-PAPC was synthesized using soybean lipoxidase and purified by HPLC on a C18 column as described [9]. All lipids were stored as stock solutions in chloroform except for diOH-PAPC, which was stored in methanol. Lipids were kept under argon or nitrogen at −20 °C. Concentrations of stock solutions were determined by phosphate assay [43]. Radiochemicals: 14C-phosphocholine was purchased from Amersham Pharmacia and [γ-32P]-ATP was from ICN. The synthesis of 4-[(n-dodecylthiomethyl)-7-(N,N-dimethylamino) coumarin (DTMAC) has been described previously [44]. PKCα isoform was from Panvera (Madison, WI). Histone HI (DTMAC) has been described previously [44]. PKCα isoform was from Panvera (Madison, WI). Histone HI was from Gibco/BRL (Grand Island, NY). All other chemicals were from Sigma or BDH.

2.2. Methods

2.2.1. Preparation of large unilamellar vesicles (LUVs)
Lipid mixtures were dried under nitrogen gas and placed under vacuum overnight to remove trace residual solvent. Lipids were then suspended in 600–800 μl 20 mM Tris, pH 7.4, 1 mM EDTA for CT assays and corresponding fluorescence measurements. For PKC assays and corresponding fluorescence measurements, lipids were suspended in 250 μl 10 mM KCl, 5 mM MgCl2, 20 mM Tris, pH 7.0. After vortexing vigorously at room temperature, the resulting lipid suspensions were freeze-thawed three or five times in liquid nitrogen, and then extruded 19 times through 0.1 μm polycarbonate filters in a Liposofast microextruder (Avestin Inc., Ottawa, ON, Canada) for CT assays and fluorescence measurements. An Avanti Mini Extruder (Avanti Polar Lipids) was used for LUV preparation for PKC assays. LUVs were used the same day for enzyme activity assays or for fluorescence measurements. Multilamellar vesicles (MLVs) used in CT assays were prepared by vigorous vortexing of dried lipids in liposome buffer.

2.2.2. CT activity assays
Rat liver CTα was expressed in Trichoplusia ni cells, using a baculovirus expression vector, and purified [32]. The assays were performed at 37 °C as described [45]. Each set of assays included a positive control sample which measured CT activity under maximally activating conditions, i.e., in the presence of 200 μM sonicated small unilamellar vesicles of egg PC: oleic acid (1/1).

2.2.3. Fluorescence measurements
LUVs were prepared by extrusion of lipid suspensions using the Avestin apparatus. The compositions of the LUVs are described in the figure legends. Fluorescence measurements were made using an SLM 4800 spectrofluorometer (SLM Aminco, Rochester, NY), with a thermoregulated sample cell holder maintained at 35 °C, or using a PTI model MP-1/PMT710 at 25 °C. The excitation wavelength for DTMAC was 397 nm, and the observed emission maxima were between 467 and 473 nm.

2.2.4. PKC activity assay
The lipid compositions of the LUVs prepared by extrusion were 30% POPS, 0–40% PAPC or diOH-PAPC, and the balance was POPC. PKC activity was measured as outlined in Mosior and Epand [46] with a few modifications. The assay was initiated by the addition of ATP and incubated at 25 °C for 10 min. The final concentrations in each assay tube were 0.8 mM CaCl2, 3 mg/ml BSA (fraction V; Sigma), 0.2 mg/ml histone, 50 ng of PKC and 10 μM (0.05 μCi) of [γ-32P]-ATP with 0.5 mg of lipid in the form of LUVs in a final volume of 250 μl. The reaction was terminated by the addition of 2 ml of ice cold 25% TCA. Samples were briefly vortexed, placed on ice and then filtered through Whatman GF/C filters. The filters were subsequently washed three times with 25% TCA, dried for 30 min at 37 °C and counted using the Cherenkov method.

2.2.5. Differential scanning calorimetry (DSC)
A Nanocal instrument from Calorimetry Sciences Corporation (American Fork, UT) was used for all scans. Films composed of DEPE and increasing mole fractions of the forms of PC were prepared by dissolving the lipids in chloroform/methanol (2:1). The films were dried in a test tube under a stream of nitrogen and then kept for 2–3 h in a vacuum dessicator. They were hydrated with Pipes buffer pH 7.4 (20 mM Pipes, 0.14 M NaCl, 1 mM EDTA and 20 mg/l sodium azide), vortexed extensively and loaded into the calorimeter sample cell. The same buffer was placed in the reference cell. Heating scan rates of 0.75 °C/min were used. The bilayer to hexagonal phase transition was fitted using parameters to describe an equilibrium with a single van’t Hoff enthalpy and the transition temperature reported as that for the fitted curve. Data was analyzed with the program Origin 5.0.

3. Results and discussion

3.1. Effects of DiOH-PAPC on the TH of membranes containing DEPE
DiOH-PAPC is a derivative of PAPC with hydroxyl groups at the 11 and 15 carbons and double bonds at C-5, C-8, and C-13 [9]. We used DSC to measure the lamellar-to-hexagonal phase transition temperature, T_H, of DEPE mixed with various mole fractions of PAPC, POPC, or diOH-PAPC (Fig. 1). To accurately measure shifts of T_H, one must add small mole fractions of lipid additive to a pure phosphatidylethanolamine, so as to maintain observable peaks in the DSC. In this study, we used DEPE as the matrix lipid. This lipid has been used for previous DSC analyses of the effects of PCs on T_H. DEPE has a T_H of 65 °C and exhibits a relatively large enthalpy for the bilayer to hexagonal transition, compared with other phosphatidylethanolamines. All three PCs raise T_H, indicating that they promote positive monolayer intrinsic curvature. However, diOH-PAPC pro-
motes the least amount of positive curvature compared with the other two forms of PC used. Lipids that stabilize the lamellar phase generate plots of mole fraction PC vs. \(T_H\) with positive slopes, whereas lipids that stabilize the inverse hexagonal phase generate negative slopes [47]. The slopes were 101 $^\circ$C/mol fraction for diOH-PAPC, 171 $^\circ$C/mol fraction for POPC, and 190 $^\circ$C/mol fraction for PAPC. We previously obtained similar results for 1-oleoyl, 2-docosahexaenoyl PC (ODPC) where the slope of \(T_H\) vs. mole fraction was 265 $^\circ$C [48]. In comparison, the slope obtained with the oxidized form of PC is considerably lower than that for several forms of PC with varying degrees of unsaturation but without OH groups. These results suggest that diOH-PAPC is a less potent bilayer stabilizer than PAPC. One would anticipate that an enzyme activity that is modulated by the degree of negative curvature strain would have greater activity in the presence of diOH-PAPC compared with PAPC.

3.2. DiOH-PAPC affects surface packing

The effect of diOH-PAPC on the interfacial polarity was assessed by analysis of effects on the fluorescence of the probe DTMAC. DTMAC is a membrane-tethered fluorescent probe whose positioning along the bilayer normal is determined when the polarity of the surrounding milieu matches the polarity of the probe’s coumarin ring. DTMAC positions preferentially in the bilayer interfacial region [44,49]. The effect of the oxidized PC on DTMAC fluorescence was investigated using the separate lipid compositions employed in the assays of PKC and CT activity. In one set of LUVs, analogous to those used in CT assays, the content of DiOH-PAPC was varied at the expense of the parent unoxidized PAPC. In the second set, analogous to the lipid compositions used in PKC assays, 30 mol% POPS was included, and the effects of 0 or 30 mol% PAPC or diOH-PAPC were examined. Increasing the mol% diOH-PAPC at the expense of PAPC resulted in a progressive decrease in DTMAC fluorescence (Fig. 2). This was also observed in the LUV systems containing POPS. This result contrasts with a lack of change in the DTMAC fluorescence intensity upon incorporation of DAG or unsaturated PEs into PC bilayers [32,36]. This decrease in fluorescence was not a result of irreversible chemical reaction between DTMAC and the hydroxylated PC, because no change in fluorescence was observed when the probe was mixed with diOH-PAPC in ethanolic solution (Table 1). There are several possible explanations for the decrease in DTMAC fluorescence: (i) it could indicate that the increasing content of diOH-PAPC creates a probe environment that is more polar. However, the decreased fluorescence was not accompanied by a red shift in the emission spectra, which is a hallmark for a more polar environment [44]. (ii) The DTMAC could be experiencing quenching from the snorkeling hydroxylated chains.

Changes in the positioning of DTMAC along the bilayer normal can be examined by measuring the degree of quenching by a doxyl group covalently linked to carbon 7 of the sn-2 chain of PC [32,36,49]. The quenching by the doxyl PC should decrease as the DTMAC moves away from the bilayer interior. Fig. 3 shows that the percentage of
quenching of DTMAC by 7-doxyl PC decreases with increasing diOH-PAPC; that is, the effect of the doxyl quencher lessens as the fraction of oxidized PC increases. Similar quenching effects were observed with 5- and 10-doxyl PC (data not shown). Moreover, when 30 mol% PAPC was replaced with 30 mol% diOH-PAPC in the LUV systems containing POPS, DTMAC fluorescence quenching by doxyl PC was reduced from 35% to 16% (Figs. 3 and 4). Thus, in both zwitterionic and anionic membrane systems, diOH-PAPC affects the behavior of DTMAC similarly. The fluorescence decrease was observed only in LUVs, not in ethanol solution (Table 1). This agrees with previous analyses showing that doxyl PCs do not directly quench DTMAC in solution [32]. Moreover, the reduction in doxyl PC quenching of DTMAC cannot be explained by immobilization of the doxyl PC, since oxidized PCs increase membrane disorder [6–9], and should increase the motional freedom of the doxyl-modified chain, hence its range along the bilayer normal. From these results, we suggest that as the diOH-PAPC content of the membrane increases, DTMAC occupies a more superficial location. The displacement might occur in a diOH-PC-enriched membrane because the packing pressure in the interfacial region—centering at the level of the glycerol backbone—becomes very high due to the tendency of the hydroxylated chains to snorkel towards the interfacial region [5,8]. In keeping with the snorkeling idea, van den Berg et al. [8] showed that when the expansion area is restrained in monolayer films, the surface pressure is much greater for peroxidized 1-palmitoyl, 2-linoleoyl PC vs. equimolar amounts of the same oxidized PC in the monolayer. These results also show that increases in negative curvature strain (i.e., as diOH-PAPC is substituted for PAPC) may not necessarily be associated with reduced interfacial packing pressure. As another example, PE, with its H-bonding headgroup, may generate higher packing pressure despite its tendency to create negative monolayer intrinsic curvature [31,32].

### 3.3. Oxidation of PAPC prevents its activation of PKC

To compare the effects of PAPC and diOH-PAPC on PKC activity, the PKC-catalyzed phosphorylation of histone was assayed in the presence of LUVs containing 30 mol% POPS and various mol% of PAPC or diOH-PAPC, with POPC as the balancing lipid. PS is a required lipid cofactor for the membrane binding and activation of PKC. In Fig. 4, PAPC or DiOH-PAPC content increased at the expense of POPC. Below 50 mol% diOH-PAPC, the lipids remained lamellar; no micelles were detected as assessed by >90% sedimentation of MLVs containing <50 mol% oxidized PCs [50]. PAPC stimulated PKC activity between 10 and 40 mol%, in keeping with previous work showing activation of PKC by unsaturated PCs [50]. However, oxidized PAPC did not. For each pair, the activity with diOH-PAPC was less than its parent phospholipid, PAPC. DiOH-PCs are stable, non-reactive species, thus the effect on PKC cannot be attributed to a chemical modification of the protein. PAPC promotes greater positive monolayer intrinsic curvature than the oxidized derivative and positive curvature agents tend to inhibit PKC. Thus, the activation of PKC by PAPC, relative to diOH-PAPC, is not through monolayer intrinsic curvature effects. Rather, the differences may be related to changes in interfacial packing pressure. There is a close parallel between the effects of PAPC vs.

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**Table 1**
The reduction in DTMAC fluorescence by diOH-PAPC and 7-doxyl PC is not observed after dilution into ethanol

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<tr>
<th>DiOH PAPC (%)</th>
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*The fluorescence of LUVs containing PAPC, the indicated percentage of diOH-PAPC, 0.5% DTMAC, with (+) or without (−) 5% 7-doxyl PC was monitored, as in the legend to Fig. 5. An aliquot of the LUVs was diluted 9-fold into ethanol and the fluorescence was measured.

Relative fluorescence intensity at the emission maximum.

b The value of the $F_{max}$ was multiplied by the dilution factor to scale to the value obtained with the LUVs.
diOH-PAPC on PKC activity and the effects on the percentage of quenching of DTMAC by doxyl-PC (Fig. 4). The effects on DTMAC suggest that the introduction of hydroxyls on the sn-2 chain of PAPC caused higher interfacial packing pressure. While the very close correlation between diOH-PAPC effects on PKC activity and on DTMAC fluorescence does not prove that the effect of this lipid on PKC is through changes in interfacial properties, it is in accord with previous studies showing PKC’s sensitivity to interfacial properties [36,50].

3.4. DiOH-PAPC activates CT in both MLVs and LUVs

Fig. 5 shows the effects of diOH-PAPC on the activity of CT, where the mol% oxidized PAPC was varied (balance PAPC) in MLVs. The results, in agreement with published data [9], demonstrate that diOH-PAPC activates CT. The activity increased between 10 and 20 mol%, reached a maximum stimulation of ~8-fold at 20 mol% oxidized component, and declined slightly at higher values. The activation between 10 and 20 mol% diOH-PAPC was apparent when the concentration of total lipid was 0.2, 0.5 or 1 mM (data not shown). The greater fold-stimulation and potency of DiOH-PAPC reported here compared to the previous report [9] can be explained in that the CT used in this study was devoid of lipid contaminants present in the preparation used previously. Fig. 6 shows the effects of 0, 15, and 25 mol% diOH-PAPC as a component of PAPC LUVs. The effects on CT of diOH-PAPC as a component of a LUV had never been examined previously. The lipid concentration for half-maximal activation of CT (AC50) can be used as a rough measure of the relative binding affinities for various lipid vesicles [32]. The AC50 was ~60 μM for vesicles with 15 mol% diOH-PAPC, and ~30 μM for vesicles with 25 mol% diOH-PAPC.
4. Conclusions

Our DSC results show that introduction of two hydroxyl groups in the sn-2 chain of PAPC decreases the ability of the lipid to raise $T_{H}$. This indicates that oxidation of the lipid generates a lipid that is less bilayer stabilizing and leads to a membrane with more negative monolayer intrinsic curvature than membranes containing the parent PAPC. Previous studies, as outlined in the Introduction, have indicated that lipid bilayers containing non-lamellar forming lipids tend to activate both CT and PKC. Hence, the effects of diOH-PAPC on CT (but not PKC) can be explained by changes in curvature strain in the membrane.

Previous studies have suggested that PKC binding to lipid bilayers is enhanced by lipid compositions that enable increased access to the hydrocarbon core [36]. DiOH-PAPC creates just the opposite effect on a membrane surface, as illustrated by the partitioning of DTMAC away from the doxyl probe, as if it were being squeezed out of the interfacial region. This increase in interfacial packing pressure can explain the lack of activation of PKC by the oxidized lipid, despite its potentially pro-active effects on monolayer intrinsic curvature. The effects of both diOH-PAPC (this work, Fig. 4) and of a series of 18:1 PEs [36] on PKC activity are better correlated with the behavior of DTMAC in membranes than with curvature strain.

In contrast with the effects on PKC, diOH-PAPC stimulates CT binding/activation. This indicates that accessibility to the bilayer core is not of as great importance for CT as for PKC, since diOH-PAPC caused a reduction in DTMAC's accessibility. A recent study also suggested a lack of correlation between CT activation by a series of di 18:1 PEs and other type II lipids and the degree of access to the hydrocarbon region [32]. With CT, the dominant factor emerging is curvature strain and not interfacial membrane properties as monitored by DTMAC. The activating effect of the oxidized PC is also linked to its effect on acyl chain order [9]. When CT’s amphipathic helix inserts into the interfacial region of a lipid bilayer, the volume available to the acyl chains of as many as 20 immediately neighboring phospholipids is increased, as well as that of the lipids in the secondary layers. Disordered acyl chains would facilitate filling the free space created by CT’s insertion, because of fewer inter-chain van der Waals interactions. The oxidized chains have less complementary surfaces for interacting with neighboring chains through van der Waals forces. Thus, there is less of an energy price associated with breaking lipid–lipid interactions to forge lipid–CT interactions (see Fig. 7). The hydroxylated chains may also present a better polarity match with the non-polar surface of CT’s amphipathic helix.

Modulation of the activity of CT and PKC provide an interesting comparison. Although these two amphitropic enzymes exhibit many similarities, the mechanism by which membrane physical properties modulate their activity is different. In the case of CT, although not the sole determinant, one of the physical properties enhancing activity is negative curvature strain. Interestingly, although many substances that promote negative monolayer intrinsic curvature also activate PKC, the mechanism for this activation appears to be less directly linked to curvature strain and is better correlated with changes in interfacial properties.

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