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Zwitterionic amphiphiles that raise the bilayer to hexagonal phase transition temperature inhibit protein kinase C

The exception that proves the rule

Richard M. Epand, Alan Stafford, Jian Wang and Raquel F. Epand

Department of Biochemistry, McMaster University, Health Sciences Centre, 1200 Main Street West, Hamilton, Ont. L8N 3Z5, Canada

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We synthesized the zwitterionic amphiphile cholesterylphosphorylethylpyridinium. This substance activated protein kinase C (PKC) in a micelle-based assay, but in a vesicle assay it was inhibitory. An analog of this compound, in which the pyridine ring is saturated and the nitrogen methylated, showed similar behaviour with PKC. Replacing cholesterol by an aliphatic alcohol lowered the extent of activation in the micelle assay. These results demonstrate that, with some membrane additives, the vesicle and micelle assays give opposite results. Results from the membrane-based vesicle assay for PKC are in accord with the generalization that zwitterionic amphiphiles that raise the bilayer to hexagonal phase transition temperature in model membranes are inhibitors of PKC

Protein kinase C; Hexagonal phase; amphiphile

1. INTRODUCTION

We have shown that a number of structurally diverse cationic amphiphiles are inhibitors of protein kinase C (PKC), while anionic amphiphiles that affect PKC activity are cofactors or activators [1]. In the case of uncharged and zwitterionic compounds, activators and inhibitors of PKC can be distinguished on the basis of their effects on lipid polymorphism, with uncharged inhibitors raising the bilayer to hexagonal phase transition temperature (T_h) while activators decrease T_h [1]. Although the molecular mechanism responsible for this correlation is not yet understood the generalization applies to a broad range of compounds. This is particularly surprising since many of the PKC activity assays were performed in a Triton-solubilzed micelle system [2] where lipid phase preference would not be expected to have an influence.

We have found that cholesterylphosphoryldimethylethanolamine is a good inhibitor of PKC and that it raises T_h [3]. In an effort to ascertain if some structural analogs of this compound are better inhibitors of PKC we synthesized a series of structurally related zwitterionic amphiphiles. All of these compounds raised T_h, but, in contradiction to our generalization, some of them activated PKC in the Triton–micelle assay. However, in a vesicle-based assay, with bilayers containing phosphatidylserine, the generalization was upheld, and all of the amphiphiles acted as inhibitors. Thus in certain cases the Triton assay does not faithfully represent results obtained in a membrane- based assay. The findings give further support for the biological relevance of lipid-phase preference for PKC activity, since this factor would be expected to be more important in an membrane system than in a micelle. Nevertheless, except for the compounds reported in this work, other modulators of PKC have qualitatively similar effects in both micelle and assays. Thus, lipidphase preference must, in general, be reflected indirectly in some property of the micelle, such as the hydrophobicity of the micelle surface. However, this is not always the case, as we demonstrate below: it is the exception that supports the applicability of this generalization to membranes.

Abbreviations: Protein kinase C, PKC; T_h, bilayer to hexagonal phase transition temperature; CPP, cholesterylphosphorylethylpyridinium; ODPP, octadecylphosphorylethylpyridinium; CPMP, cholesteryl-phosphorylethyl-*N*-methyl piperidinium; ODPC, octadecylphosphorylcholine; PS, phosphatidylserine.

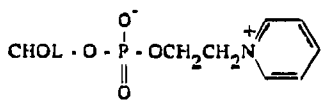
Correspondence address: R.M. Epand, Department of Biochemistry, McMaster University, 1200 Main Street West, Hamilton, Ont. L8N 3Z5, Canada.

2. EXPERIMENTAL

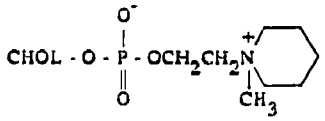
2.1. *Materials*

All phospholipids were purchased from Avanti Polar Lipids (Pelham, AL) and were pure as determined by TLC. Histone (type III-S) and bovine brain D-sphingosine were purchased from Sigma Chem. Co. (St. Louis, MO). [γ-^{32}P]Adenosine 5'-triphosphate (3 Ci/μmol) was from NEN (Montréal, Québec). Ultra-pure grade $Mg(NO_3)_2$ was

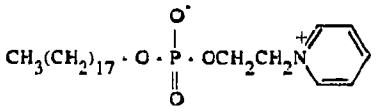
TABLE I



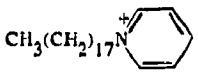
CPP



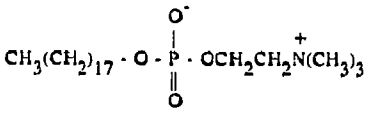
CPMP



ODPP



ODPI

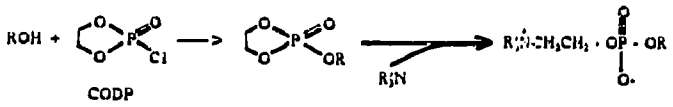


ODPC

obtained from Alfa Chem. Co. Ultra-pure grade Tris was obtained from BRL. PKC was purified from rat brain as previously described [4]. Double-distilled water was obtained from a Gelman Science Water I filter.

2.2. *Synthetic amphiphiles*

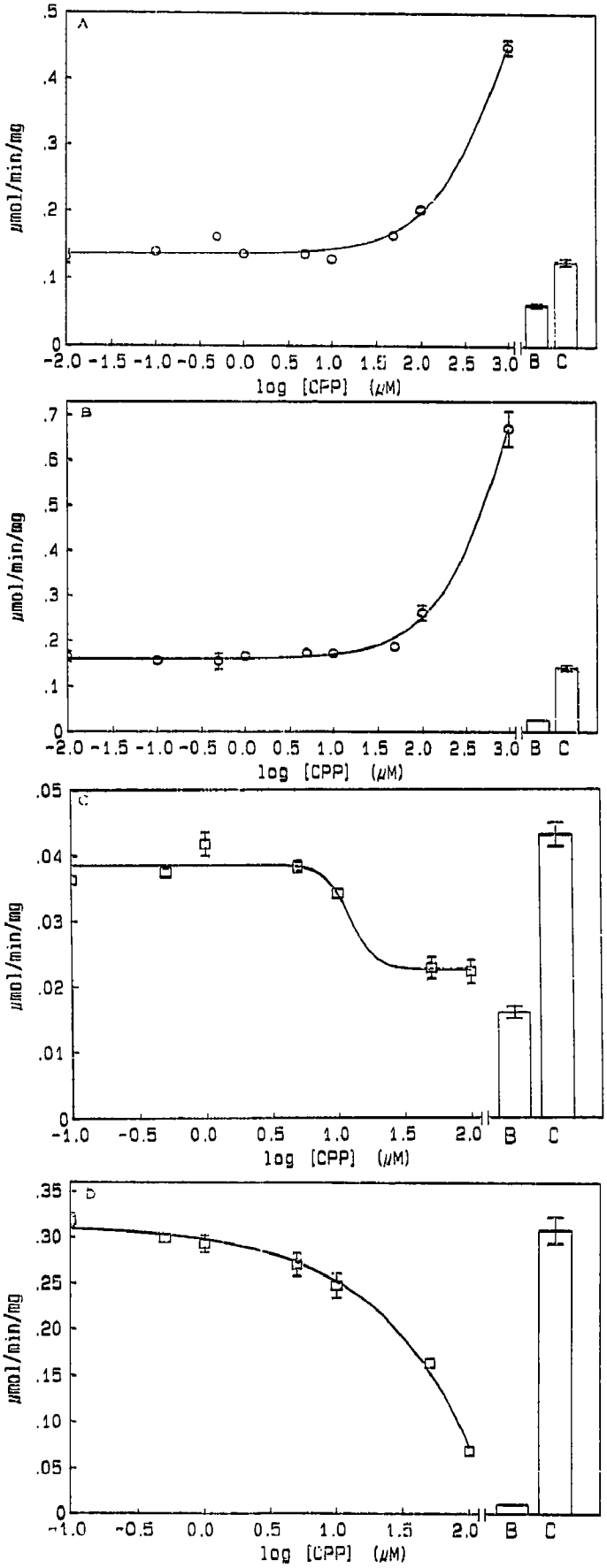
The structure of the synthetic amphiphiles used in this work are given in Table I. The synthesis of these amphiphiles follows Scheme I, which utilizes the reactivity of 2-chloro-2-oxo-1,3-dioxaphospholane (CODP). This reagent reacts with primary alcohols to give an organic phosphoric ring compound as first shown by Chabrier [5]. The intermediate is reactive toward nucleophiles and has been widely used in the preparation of phospholipids in high yield [6].



Scheme I

2.3. *CODP*

All reagents used in the synthetic work were purchased from Aldrich Chem. Co. unless otherwise specified. To a solution of phosphorous trichloride in anhydrous dichloromethane (25 g/40 ml), redistilled ethylene glycol (10 ml) was added drop-wise with stirring. After the addition was complete the dichloromethane was removed by distillation at atmospheric pressure. The residue was then distilled under reduced pressure (12–15 mm Hg) at 43–45°C (lit. 45.5–47°C) to give 2-chloro-1,3,2-dioxaphospholane [7]. The product (18 g) was dissolved in 40 ml dry toluene, and dry oxygen was passed through the solution until the exothermic reaction subsided. The solvent was removed on a rotovaporator. The product was then distilled at 0.4–0.5 mm Hg at 75–80°C to give 2-chloro-2-oxo-1,3-dioxophospholane (CODP) [6].



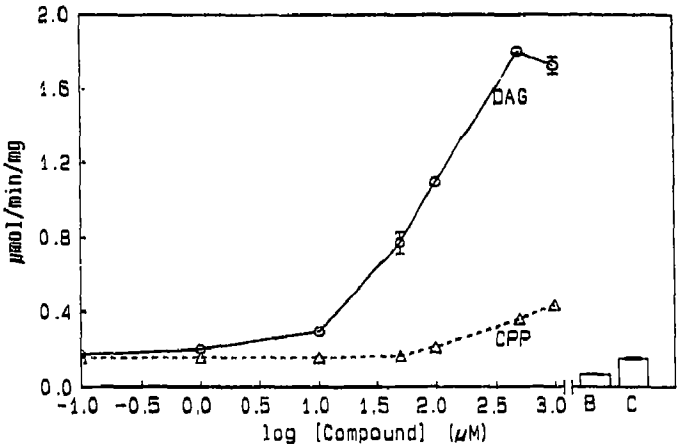


Fig. 2. Comparison of CPP and diolein (DAG) stimulation of PKC in the micelle assay. B, background without PS; C, control with PS but no CPP or diolein.

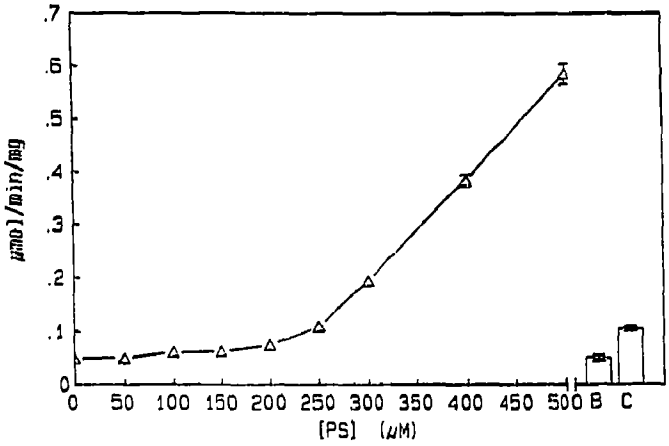


Fig. 3. Phosphatidylserine dependence of the activation of PKC by CPP in the micelle assay. CPP concentration was held at 500 μM. B, background in the absence of PS and CPP; C, control with 400 μM PS and no CPP.

Reaction of alcohols with CODP, followed by reaction with tertiary amines (see Scheme 1), was based on the procedure of Bhatia and Hajdu for phospholipid synthesis [8]. A solution of CODP in dichloromethane (1.14 g/8 ml) was added drop-wise with stirring to a solution of cholesterol (1.93 g) and triethylamine (0.81 g) in 20 ml dichloromethane. The addition occurred over a period of 2 h with the temperature maintained between 0 and 5°C. The solution was slowly warmed to room temperature, and the solvent removed under vacuum. The product was dissolved in 50 ml tetrahydrofuran, and the insoluble residue discarded. The solvent was evaporated to give the solid cholesteryl-2-oxo-1,3-dioxaphospholane. An analogous procedure was used for the alkyl derivatives, with 1-octadecanol replacing cholesterol to yield octadecyl-2-oxo-1,3-deoxaphospholane.

2.4. *CPP*

Cholesteryl-2-oxo-1,3-dioxaphospholane (0.36 g) was dissolved in 15 ml acetonitrile containing 3 ml pyridine. The misxture was refluxed for 72 h after which a white powder precipitated. The precipitate was filtered off, washed with acetonitrile, and dissolved in chloroform:methanol (4/1, v/v). The solution was extracted with water and dried over sodium sulphate. The solvents were then removed on a rotovaporator. The product was purified by chromatography on silica gel. It melted at 228°C with decomposition. The product, as well as those described below, were characterized by mass spectroscopy and by ^{1}H NMR.

2.5. *ODPP*

Starting with 1-octadecanol, instead of cholesterol, CODP was reacted to form the 2-octadecyl-2-oxo-1,3-dioxaphospholane, followed by reaction with pyridine to give the final product, ODPP.

2.6. *CPMP*

As for CPP except that *N*-methyl piperidine was used to react with the phospholane.

2.7. *ODPC*

As for ODPP except trimethylamine was used instead of pyridine.

2.8. *ODPI*

1-Iodooctadecane (1 g) was dissolved in 5 ml pyridine and the solution heated between 60 and 70°C with stirring for 18 h. The solvents were removed on a rotovaporator. The residue was washed with hexane containing a trace of methanol. The product was recrystallized from chloroform/hexane and gave a melting point of 103°C in agreement with literature values [9].

2.9. *Micelle assay for PKC*

This assay was adopted from the procedure of Bell and coworkers [2]. Briefly, 4.75 mM Triton X-100 (0.3%) containing 8 mol % bovine brain phosphatidylserine in the presence or absence of 2 mol % 1,2-diolein and/or synthetic amphiphile. The assay solution contained 20 mM Tris–HCl, 10 mM $MgCl_2$, 200 μg/ml histone III-S, 80 μg/ml dithiothreitol, 200 μg $CaCl_2$ or 2 mM EDTA and 10 μM [γ-^{32}P]adenosine 5'-triphosphate (0.5 μCi). After addition of PKC the reaction mixture was incubated for 10 min at 30°C and then stopped with trichloroacetic acid and BSA, as previously described [3].

2.10. *Vesicle assay for PKC*

This assay was adopted from the procedure of Boni and Rando [10]. Sonicated vesicles were prepared containing 1-palmitoyl-2-oleoylphosphatidylcholine and 1-palmitoyl-2-oleoylphosphatidylserine in a 4:1 molar ratio. In some cases, the vesicles also contained 17 mol % diolein and/or variable quantities of synthetic amphiphile. Total phospholipid concentration was 50 μM. The assay procedure was the same as that described for the micelle assay except that 20 μM ATP and 1 mg/ml histone were used.

2.11. *Differential scanning calorimetry (DSC)*

Lipid films of 1,2-dielaidoylphosphatidylethanolamine containing varying amounts of synthetic amphiphile were deposited from solutions of chloroform:methanol (2:1, v/v). The dried lipid films were hydrated in an aqueous buffer at pH 7.40 containing 20 mM PIPES, 1 mM EDTA, 150 mM NaCl and 0.02 mg/ml NaN_3. The suspension was vortexed vigorously for 30 s at 45°C, the samples degassed, and loaded into a Microcal MC-2 DSC instrument. A heating scan rate of 0.7 degrees/min was employed. Data were acquired and analyzed using Microcal software.

←

Fig. 1. Effect of CPP on PKC. (A) Micelle assay in absence of diolein. (B) Micelle assay in the presence of 2 mol % diolein. (C) Vesicle assay in the absence of diolein. (D) Vesicle assay in the presence of 14 mol % diolein. B, background in the absence of PS and diolein but containing Triton X-100 for the micelle assay; C, control containing PS and diolein, where indicated, but no CPP.

Table II

Characteristics of zwitterionic modulators of PKC

Compound[a]	Slope[b] (deg/mol fraction)	PKC activity[c]	
		Micelle assay	Vesicle assay
CPP	314 ± 49	activate	inhibit
ODPP	272 ± 25	biphasic[d]	inhibit
CPMP	226 ± 6	activate	inhibit
ODPC	120 ± 13	no effect	inhibit
ODPI[e]	240 ± 10	inhibit	inhibit

[a] See Table I for structures
[b] Slope of a plot of T_h vs. mol fraction of compound in phosphatidylethanolamine
[c] Assays in presence of diolein.
[d] Weakly stimulatory to 100 μM then inhibitory at 1 mM
[e] Cationic amphiphile

3. RESULTS

All of the amphiphiles raised T_h and all inhibited PKC in the vesicle assay in the presence of diolein (Table II). Concentrations of between 10 and 100 μM amphiphile were required for inhibition with the higher concentrations resulting in complete inhibition of enzyme activity. In the micelle assay, however, the effects were varied. CPP and CPMP at concentrations between 100 μM and 1 mM activated above the level given by diolein alone. The potency of the amphiphiles in the two assay systems cannot be compared because, in the micelle phase, they are diluted more with Triton than they are diluted with phospholipid in the vesicle assay. CPP activates in the micelle assay both in the presence and absence of diolein, but inhibits in the vesicle assay in both of these conditions (Fig. 1). Even though CPP can activate PKC above the level exhibit than diolein as an activation (Fig. 2). Nor can CPP activate PKC in the absence of phosphatidylserine (Fig. 3).

4. DISCUSSION

Detergents, such as Triton X-100, will raise T_h. Therefore, they should be inhibitors of PKC, yet the activity of this enzyme is supported in micelles containing a high concentration of certain detergents [2]. In addition, micelle-forming amphiphiles, such as sodium salts of fatty acids [11] or short-chain phosphatidylcholines [12], can activate PKC in a Ca^{2+}, phosphatidylserine-independent manner. It is not known why some detergent micelles support the activity of PKC better than others, nor why the activity is dependent on Ca^{2+} and phosphatidylserine in some cases and in others not. The amphiphiles used in the present work are not potent modulators of PKC: they affect the activity of PKC in the micelle assay when present at 5-50% of the concentration of the detergent Triton X-100. At these high concentrations our synthetic amphiphiles are undoubtedly affecting the properties of the detergent micelle. The resulting effect of these changes in micelle properties on PKC activity is not necessarily related to changes observed in a membrane system. Thus, CPP and CPMP activate PKC in micelles but inhibit it in a vesicle assay. Results from the vesticle assay are in accord with the generalization that zwitterionic substances than raise T_h inhibit PKC [1].

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