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The role of hydrophobic interactions in the phospholipid-dependent activation of protein kinase C

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The effects of hydrophobic interactions on the activation of Ca^{2+}-stimulated phospholipid-dependent protein kinase (protein kinase C), isolated from mouse brain, by phosphatidylserine (PS) and diacylglycerol (DAG) or phorbol 12-myristate 13-acetate were studied. To maintain bilayer structure during assay conditions, phosphatidylcholine was added to the PS vesicles. The vesicular structure of all types of PS was confirmed by freeze-fracture electron microscopy. The PS-dependent activation of purified protein kinase C from mouse brain is affected by the fatty acid composition of PS: an inverse relationship between the unsaturation index of PS (isolated from bovine heart, bovine spinal cord or bovine brain) and the ability to activate protein kinase C was demonstrated. In highly saturated PS lipid dispersions, only slight additional activation of protein kinase C by DAG was found, in contrast with highly unsaturated PS lipid dispersion, where DAG increased protein kinase C activity by 2–3-fold at optimal PS concentrations. We quantified the formation of the protein kinase C–Ca^{2+}–PS–phorbol ester complex by using [^{3}H]phorbol 12,13-dibutyrate ([^{3}H]PDBu). The efficiency of complex-formation, determined as the amount of [^{3}H]PDBu bound, is not affected by variations in the hydrophobic part of PS. These results indicate a role of the hydrophobic part of the activating phospholipid in the activation mechanism of protein kinase C and in the action of cofactors.

INTRODUCTION

The Ca^{2+}-activated phospholipid-dependent protein kinase (protein kinase C) has been implicated as a regulatory element in receptor-mediated signal transduction, and as the major cellular receptor for, and mediator of the action of, tumour-promoting phorbol esters [1,2]. During signal transduction induced by ligand–receptor binding, phosphoinositides are hydrolysed in the plasma membrane and diacylglycerol (DAG) is transiently generated [3–5]. DAG and the biologically active phorbol ester, phorbol 12-myristate 13-acetate (PMA), increase the affinity of protein kinase C for phospholipids and for Ca^{2+} ions to the physiological range. As a result, the predominantly cytoplasmic protein kinase C becomes associated with the plasma membrane on incubation with PMA or DAG-generating polypeptides and is fully activated by interaction with the phospholipids in the plasma membrane at physiological Ca^{2+} concentrations [4,6–8].

The presence of negatively charged phospholipids such as phosphatidylserine (PS), phosphatidylinositol (PI) or phosphatidic acid (PA) in the membrane is required for activation of protein kinase C [9–12]. *In vitro*, protein kinase C can be activated by negatively charged phospholipids such as PS, PI and PA, not by neutral phospholipids such as phosphatidylcholine (PC) or phosphatidylethanolamine (PE) [1,11]. Addition of DAG or PMA increases protein kinase C activity *in vitro* [8] and its association with PS-containing lipid bilayers [13].

The morphology of the lipid aggregates (i.e. large or small unilamellar vesicles, multilamellar vesicles or micelles) has been demonstrated to cause differential activation of protein kinase C [14,15], indicating a possible regulatory role for the lipid organization of the plasma membrane in the process of activation. This has been further confirmed by Cabot [16], who demonstrated that binding of phorbol esters to human promyelocytic leukaemia cells is modulated by variations in the phospholipid composition of the plasma membrane.

Protein kinase C contains two functionally distinct domains: a hydrophobic phospholipid-, phorbol ester- and DAG-binding domain, and a hydrophilic catalytic domain [17]. Through Ca^{2+}-dependent proteinases, the native enzyme generates a catalytically active phospholipid-independent form of the enzyme (protein kinase M; 50 kDa [18–20]) and a phorbol ester- or DAG-binding 38 kDa fragment [17]. The interaction of the hydrophobic domain with a lipid bilayer is thus responsible for the phospholipid-dependent activation.

On the basis of experiments with mixed micelles, a model of protein kinase C activation by PS, DAG and Ca^{2+} has been formulated, in which an electrostatic complex is formed between one molecule of protein kinase C, one Ca^{2+} ion, one DAG molecule and at least four PS molecules [21]. However, little is known about the nature of the interactions between PS, protein kinase C, DAG or PMA and Ca^{2+} in the activated complex, either hydrophilic or hydrophobic interactions. Structural studies of DAG have shown that the length and the unsaturation of the fatty acid chains affect its function as

Abbreviations used: PMA, phorbol 12-myristate 13-acetate; PDBu, phorbol 12,13-dibutyrate; DAG, diacylglycerol; PS, phosphatidylserine; PS-SC, phosphatidylserine from bovine spinal cord; PS-BB, phosphatidylserine from bovine brain; PS-BH, phosphatidylserine from bovine heart; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PA, phosphatidic acid.

cofactor in the phospholipid-dependent activation of protein kinase C [22–24].

These considerations have led to the hypothesis that the structural features of the hydrophobic domains of activating phospholipids might affect the activation of protein kinase C either by interaction with the enzyme or by an effect on the nature of the lipid bilayer.

In this study we describe experiments *in vitro* to investigate the interaction between purified protein kinase C and unilamellar phospholipid bilayers. We demonstrate that variations in the hydrophobic part of the activating phospholipids affect both the lipid-dependent activation of protein kinase C and the action of the cofactor DAG.

EXPERIMENTAL

Purification of protein kinase C

Protein kinase C was purified from mouse brain essentially as described for bovine brain [25]. Mouse brains were homogenized in 20 mM-Tris (pH 7.5)/2 mM-EDTA/10 mM-EGTA and centrifuged for 90 min at 21500 rev./min in a Beckman SW27 rotor. The supernatant was applied to a DEAE-cellulose DE52 column, equilibrated with 20 mM-Tris (pH7.5)/2 mM-EDTA/5 mM-EGTA/50 mM-β-mercaptoethanol. The enzyme was eluted by application of a linear NaCl concentration gradient (0–0.4 M) in the same buffer. The fractions containing protein kinase C activity were pooled, concentrated and adjusted to 18% (w/v) in $(NH_4)_2SO_4$; after standing for 5 min at 4 °C, the suspension was centrifuged for 20 min at 10000 *g* and the supernatant was applied to an octyl-Sepharose column, equilibrated in 20 mM-Tris buffer (pH 7.5)/2 mM-EGTA/50 mM-β-mercaptoethanol containing 1 M-$(NH_4)_2SO_4$. Protein kinase C was eluted with the same buffer containing 0.2 M-$(NH_4)_2SO_4$ and subsequently applied to a Sephadex G-100 column equilibrated in 20 mM-Tris (pH 7.5)/0.5 mM-EGTA/50 mM-β-mercaptoethanol. Elution was carried out with the same buffer, and the fractions containing protein kinase C were pooled and concentrated. NaCl was added to the concentrated fraction to a final concentration of 3 M, and it was applied to a phenyl-Sepharose CL4B column equilibrated in 20 mM-Tris (pH 7.5)/2 mM-EDTA/50 mM-β-mercaptoethanol/5% sucrose/3 M-NaCl. The column was washed with the same buffer containing 0.5 M-NaCl, and protein kinase C was eluted with the buffer without NaCl.

Sucrose and Triton X-100 were added to final concentrations of 25% and 0.01% respectively. The final preparation is a 81 kDa protein, essentially pure as demonstrated by silver staining of a polyacrylamide gel.

Protein kinase C assay

The Ca^{2+}- and phospholipid-dependent protein kinase activity was assayed by adding 0.01–0.03 μg of purified protein kinase C to a reaction mixture (final volume 125 μl) consisting of 20 mM-Tris, pH 7.5, 7.5 mM-magnesium acetate, leupeptin (10 μg/ml), histone IIIs (200 μg/ml), 10 μM-ATP, vesicles containing PS and/or PC as indicated and diolein (3.2 μg/ml) and 0.5 mM-$CaCl_2$. The control value was determined in the same reaction mixture without PS, diolein and/or $CaCl_2$. The mixtures were preincubated for 5 min at 30 °C. The reaction was started by the addition of ATP and [γ-^{32}P]ATP (10 μM; 2×10^6 c.p.m. per incubation) and allowed to proceed at 30 °C for 5 min. The reaction was stopped by addition of 1 ml of ice-cold 25% trichloroacetic acid. The precipitated protein was collected on a Millipore filter (0.45 μm), and washed with 5×3 ml of ice-cold 10% trichloroacetic acid containing 10 mM-$Na_4P_2O_7$.

Preparation of unilamellar vesicles

Phospholipids and diolein were mixed and dried under nitrogen. The lipids were resuspended in 20 mM-Tris/HCl, pH 7.5, and tip-sonicated at 20 °C under N_2 for 3 min (Kontes sonifier, power 2.5, tune 2.5).

To prepare the vesicles for electron microscopy, the suspension was centrifuged at room temperature for 1 h at 40000 rev./min in a Beckman SW50 rotor. Samples (0.2 μl) of the pellet were rapidly frozen between copper sandwiches by dipping into liquid propane. A standard freeze-fracture procedure was carried out in a Cryofact 190 (Reichert Jung, Paris, France). Replicas mounted on copper grids (type Polaron Hex 700 TB) were examined in a Zeiss EM-10 electron microscope.

[^{3}H]PDBu–protein kinase C–lipid complex

Binding of [^{3}H]PDBu to protein kinase C was performed as described by Tanaka *et al.* [26].

Materials

PS-SC was purchased from Lipid Products (Redhill, Surrey, U.K.); diolein and PC (egg lecithin) were from Sigma. PS-BH and PS-BB were a gift from Dr. A. Rietveld (University of Utrecht, Utrecht, The Netherlands). The isolation and characterization procedure has been described [27,28]. The PS preparations were pure and essentially Ca^{2+}-free. [γ-^{32}P]ATP was purchased from Amersham, and [^{3}H]PDBu from NEN.

RESULTS

Protein kinase recovery from control- and PMA-treated cells in the presence and the absence of Ca^{2+}

The recovery of protein kinase C from different cellular compartments with different extraction methods permits comparison of the lipid-bilayer–protein kinase C interaction in control and PMA-stimulated cells. Protein kinase C in HeLa cells was found predominantly in the cytoplasmic fraction when the cells were lysed in a buffer containing Ca^{2+} chelators (EGTA and EDTA). However, when the cells were lysed without Ca^{2+} chelators in the presence of 0.01 mM- or 1 mM-$CaCl_2$, more than 90% of the total protein kinase C was found in the particulate fraction, indicating a Ca^{2+}-dependent association with the particulate fraction of the cells. Washing the particulate fraction with an EGTA/EDTA-containing buffer resulted in the removal of protein kinase C from the particulate fraction.

After treatment of HeLa cells with PMA, most protein kinase C activity was found in the particulate fraction, independent of the presence of Ca^{2+} chelators or Ca^{2+} during lysis. Protein kinase C could be recovered from the particulate fraction of PMA-treated cells by extraction with 1% Nonidet P40 or 1% Triton X-100.

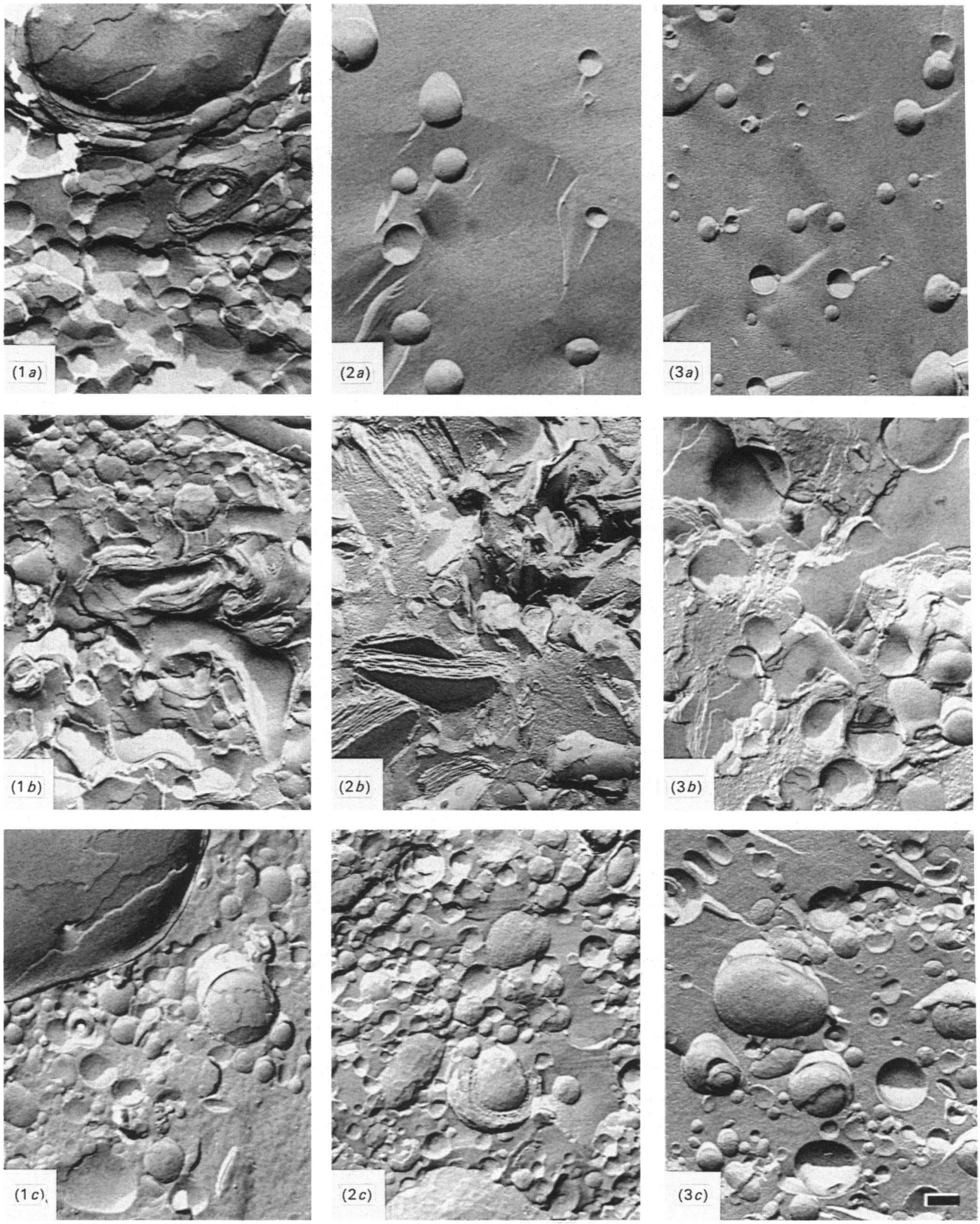

Fig. 1. Electron-microscopic analysis of liposomes of variable PS types and PS/PC compositions

See the Experimental section for detailed information on methods used. Columns: 1, PS isolated from bovine heart (PS-BH); 2, PS isolated from bovine spinal cord (PS-SC); 3, PS isolated from bovine brain (PS-BB). Rows: *a*, 100% PS in 20 mM-Tris buffer, pH 7.5; *b*, 100% PS + protein kinase C-activity assay mixture; *c*, 25% PS + 75% PC + protein kinase C-activity assay mixture. The black bar represents 100 nm.

These results indicate that PMA induced a Ca^{2+}-chelator-insensitive interaction between protein kinase C and phospholipids in the particulate fraction. The changed nature of the protein kinase C–plasma-membrane interaction could be due to an increased affinity of the protein kinase C–PMA–lipid complex for Ca^{2+}, to an interaction between protein kinase C and the hydrophobic core of the plasma membrane, or to both. Both an increased affinity for Ca^{2+} [8] and indications for an increased hydrophobic interaction [13] in the presence of PMA have been demonstrated. Furthermore, the complex of purified protein kinase C, PS liposomes and Ca^{2+} in the absence of DAG/PMA is also insensitive to Ca^{2+} chelators, but can be efficiently separated by 1% Triton X-100 or 1% Nonidet P40 (results not shown).

Since the interaction of phospholipids with protein kinase C is responsible for the activation of the enzyme, we investigated whether variations in the hydrophobic part of the phospholipid affected the activation of protein kinase C. For this purpose we had available three different types of PS, which varied in the degree of saturation of the fatty acid chains. Two of the PS types were isolated from bovine brain and bovine heart [27,28], and the other was the commercially available product isolated from bovine spinal cord.

In Table 1 the fatty acid compositions of the three PS types and their unsaturation indices are given. The unsaturation index is defined as the number of unsaturated carbon bonds per 100 fatty acid molecules. PS from bovine heart (PS-BH) has the highest number of unsaturated carbon bonds (112/100 molecules), PS from bovine brain (PS-BB) the lowest (50/100 molecules) and the commercial PS from bovine spinal cord (PS-SC) a value in between (90/100 molecules).

Vesicles of PS in combination with PC: morphology

It was our intention to study the PS-dependent protein kinase C activation under conditions where a (unilamellar) bilayer structure was maintained.

The three PS types formed large unilamellar vesicles (LUV's) after sonication, as analysed by freeze-fracture electron microscopy (Fig. 1, panels 1*a*, 2*a* and 3*a*). However, addition of Ca^{2+}, Mg^{2+} and other compounds used in the activity assay resulted in the complete disappearance of the vesicle structure, and multilamellar close-packed sheets of phospholipid were formed (Fig. 1, panels 2*a*, 2*b* and 2*c*). When 50% or more (w/w) PC was added to the lipid mixture [29,30], the liposomes retained their vesicular character (shown for 25% PS/75% PC; Fig. 1, panels 1*c*, 2*c* and 3*c*). Addition of diolein or PMA under these conditions had no effect on the morphology of the lipid dispersion (results not shown). Liposomes composed of 25% PS/75% PC (w/w) were used to analyse the lipid-dependent activation of protein kinase C.

Table 1. Fatty acid composition and unsaturation index of three types of PS

The fatty acid composition was determined by g.l.c. of the fatty acid methyl esters and is expressed as mol%.

Fatty acid	PS isolated from Bovine heart*	Bovine spinal cord	Bovine brain*
$C_{16:0}$	1	1	1
$C_{16:1}$	–	1	–
$C_{18:0}$	56	42	51
$C_{18:1}$	16	40	45
$C_{18:2}$	6	–	–
$C_{18:3}$	–	14	–
$C_{20:1}$	–	–	2
$C_{20:3}$	2	–	1
$C_{20:4}$	17	2	–
$C_{20:5}$	2	–	–
Unsaturation index	112	90	50

* Taken from reference [28].

Analysis of PS-dependent activation of protein kinase C

Protein kinase C (0.03 μg) was optimally activated by a PS concentration of 192 μg/ml for all three PS types (Fig. 2, open symbols). However, the maximal activation of protein kinase C for the three PS types appeared to be dependent on the degree of saturation of PS.

The maximal activation is induced by PS-BB, followed by PS-SC and PS-BH; the maximal activation is inversely proportional to the unsaturation index of the activating phospholipid.

Analysis of the kinetics of the interaction between protein kinase C according to Hanes, i.e. the relationship between [PS] and [PS]/specific activity of protein kinase C, resulted in three linear curves (Fig. 3, open symbols).

In the Hanes equation:

$$\frac{[PS]}{SA} = \frac{[PS]}{SA_{max.}} + \frac{K_s}{SA_{max.}}$$

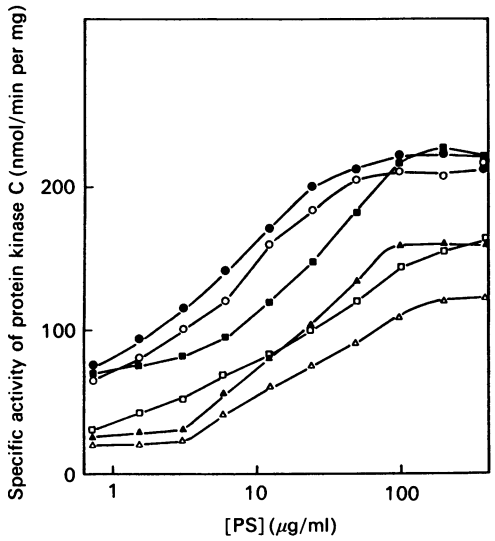

Fig. 2. PS-concentration-dependent activation of purified protein kinase C in the absence (open symbols) and the presence (closed symbols) of diolein

Diolein (final concn. 3.2 μg/ml) was added in dimethyl sulphoxide to the sonicated vesicles, giving a final concentration of 0.3% dimethyl sulphoxide in the vesicles. The same dimethyl sulphoxide concentration was added to the samples of PS without diolein. Key: ▲, △, PS-BH; ■, □, PS-SC; ●, ○, PS-BB. See the Experimental section for detailed information. Values are means of duplicates and of three (PS-BB and PS-SC) or two (PS-BH) experiments.

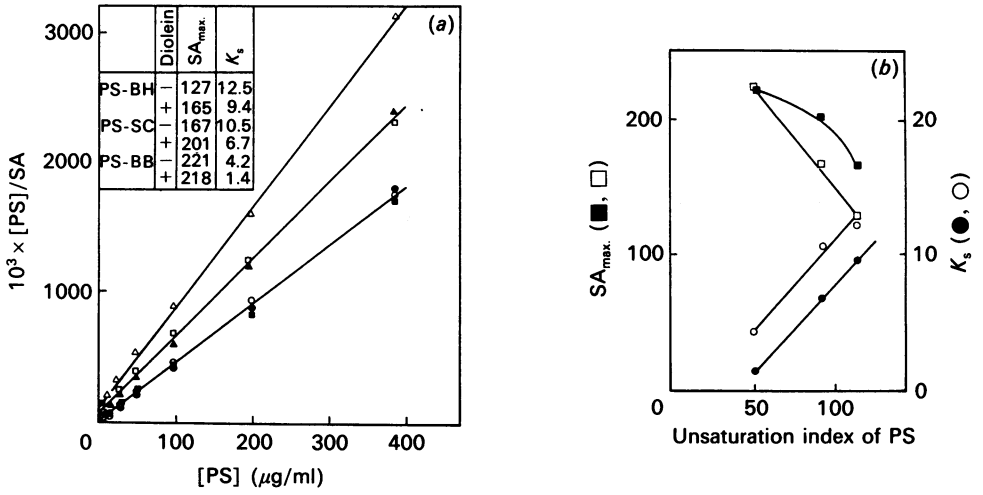

Fig. 3. PS-dependent activation of protein kinase C

(*a*) Hanes analysis. K_s and $SA_{max.}$ were calculated by using the Hanes equation:

$$\frac{[PS]}{SA} = \frac{[PS]}{SA_{max.}} + \frac{K_s}{SA_{max.}}$$

Key: △, PS-BH, no diolein; ○, PS-BB, no diolein; □, PS-SC, no diolein; ▲, PS-BH+diolein; ●, PS-BB+diolein; ■, PS-SC+diolein. (*b*) Schematic relation between the unsaturation indices of the PS types, the $SA_{max.}$ and the K_s values as calculated with the Hanes equation in the absence (○, □) or the presence (●, ■) of diolein.

where K_s is the dissociation constant of the PS–protein kinase C complex and $SA_{max.}$ the maximal specific activity of protein kinase C. With this equation we calculated the K_s and $SA_{max.}$ for the reaction between protein kinase C and the three PS types (insert, Fig. 3).

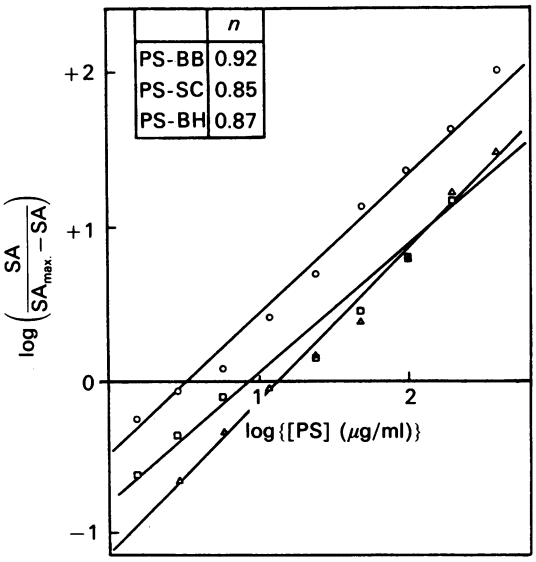

Fig. 4. Hill plots calculated with the results from Figs. 2 and 3

n (the number of PS molecules per enzyme molecule) is calculated from the Hill formula by using $SA_{max.}$ from the Hanes calculations:

$$\log\left(\frac{SA}{SA_{max.}-SA}\right) = n \cdot \log[PS] - \log K_s$$

Key: △, PS-BH; □, PS-SC; ○, PS-BB.

The Hanes equation yielded the same conclusion as Fig. 2: maximum PS-dependent activation is inversely proportional to the unsaturation index of the PS. On the other hand, the dissociation constant (K_s) is proportional to the unsaturation index (Fig. 3*b*, open symbols). To calculate the number of PS molecules reacting with one protein kinase C molecule, we used the Hill equation:

$$\log[SA/SA_{max.} - SA)] = n \cdot \log[PS] - \log K_s$$

In this equation *n* is the number of PS molecules reacting with one protein kinase C molecule and K_s is the dissociation constant. By using $SA_{max.}$ from the Hanes plots, the *n* values for all three PS types were calculated to be approx. 1 (Fig. 4, and insert). These results lead to the conclusion that protein kinase C is differentially activated by PS types that vary in their fatty acid composition, i.e. the hydrophobic part of the molecule. This indicates that the hydrophobic interaction between phospholipid and protein kinase C does affect the activation of the enzyme.

Analysis of cofactor action in protein kinase C activation by PS-BB, PS-BH and PS-SC

Cofactors (activators) of protein kinase C, phorbol esters and DAG are thought to function by increasing the affinity of protein kinase C for Ca^{2+} and PS, but the nature of the interaction between the enzyme, phospholipid and the cofactor is not clear.

We analysed the effects of diolein on the PS-dependent activation of protein kinase C under the conditions described above. Optimal activation was induced at a diolein concentration of 3.2 μg/ml. Increasing the diolein concentration did not increase the enzyme activity (results not shown).

Addition of diolein to vesicles of PS-BH and PS-SC increased the protein kinase C activation in the PS concentration range used. However, addition of diolein

to PS-BB vesicles hardly affected activation (Fig. 2, closed symbols).

The Hanes plot in all three cases was linear (Fig. 3, closed symbols) and yielded new values for $SA_{max.}$ and K_s (insert in Fig. 3). $SA_{max.}$ for protein kinase C with PS-BH and PS-SC increased after addition of diolein; the $SA_{max.}$ with PS-BB was hardly affected by diolein (as already concluded from Fig. 2).

On the other hand, the dissociation constants (K_s) of the three PS–protein kinase C complexes were affected in an essentially identical way: in each case the absolute value of K_s is decreased. However, the relative effect of diolein on the K_s values is different for the three PS types (for PS-BH, PS-SC, PS-BB respectively, a factor of 1.3, 1.6 or 3.0), indicating that the diolein effect on PS–protein kinase C interaction involves an effect on the hydrophobic interaction between the enzyme and the activating phospholipid.

Finally, we have checked the possibility of a variable capacity of the three PS types to form lipid–protein kinase C–Ca^{2+}–DAG complexes.

The experiments shown in Fig. 3 were repeated with PMA (50 ng/ml) instead of diolein with quantitatively and qualitatively the same results (not shown), indicating that the effects of DAG and PMA on hydrophobic interaction between PS and protein kinase C are comparable.

It has been demonstrated that the stoichiometry of PS–protein kinase C–Ca^{2+} and phorbol ester or DAG is constant [15,20,22]. Our calculations of n from the Hill plots indicate the same. Thus the amount of labelled phorbol ester ([^{3}H]PDBu) found in the complexes should be proportional to the number of protein kinase C and phospholipid molecules and independent of the type of PS.

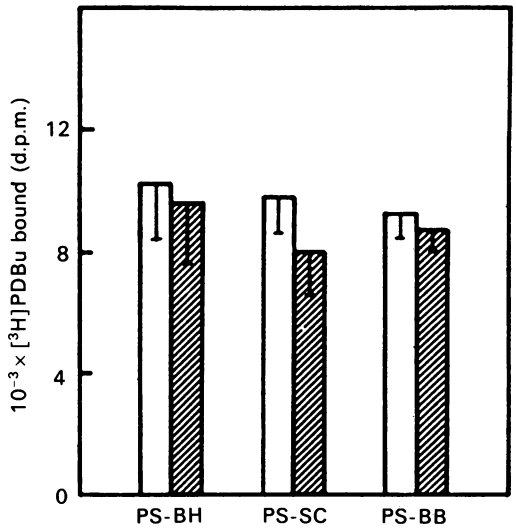

Fig. 5. [^{3}H]PDBu–protein kinase C–lipid–Ca^{2+} complex-formation of protein kinase C and lipid vesicles made of various PS types

The values have been corrected for the control values of complex-formation in the absence of Ca^{2+} and lipid or in the presence of 10 μM unlabelled PDBu. Values are the means of duplicates in two experiments; the S.D. is indicated in the bars. □, 100% PS; ▨, 25% PS/75% PC (w/w).

We have determined [^{3}H]PDBu binding in mixtures of protein kinase C, Ca^{2+} and liposomes of varied compositions. When comparing the complex-forming capacity of the three PS types, we found little difference between them irrespective whether 100%-PS liposomes (Fig. 5, □) or 25% PS/75% PC-liposomes were used (Fig. 5, ▨). The rather high error rate, owing to the high non-specific binding of [^{3}H]PDBu, prevents an exact quantification of the complex-formation. However, analysis of variance of the data showed no significant difference in the 5%-significance levels in LSD and t tests. The results, however, indicate that binding of an identical number of [^{3}H]PDBu molecules to a constant number of protein kinase C and PS molecules leads to variable extents of protein kinase C activation, dependent on the fatty acid composition of the activating phospholipid.

DISCUSSION

Both hydrophilic and hydrophobic interactions are involved in the activation of protein kinase C. Ca^{2+} ions are involved in the hydrophilic association of protein kinase C with the negatively charged phospholipids in the plasma membrane or an artificial bilayer.

The present results have demonstrated a role for hydrophobic interactions in the activation of protein kinase C by phospholipid. Hydrophobic interactions could involve the actual lipid–protein interactions and could affect the partition of activators of protein kinase C (PMA/DAG) in the lipid bilayer. These two issues can be separated in the experiments comparing the PS-dependent activation of protein kinase C in the absence and the presence of DAG. Variations in the hydrophobic part of the activating phospholipid, PS, are responsible for the induction of different maximal specific activities and dissociation constants for the PS–protein kinase C complexes in the absence of DAG or PMA. These effects should be due to actual lipid–protein kinase C interactions. The DAG-dependent activation of protein kinase C, relatively increased in highly unsaturated PS, cannot be attributed to either of the possible events, to protein–lipid interactions or to an effect on the partition of the activator into the bilayer.

Previous findings by our group that the insertion of protein kinase C in a PS-containing lipid bilayer is increased after addition of diolein or PMA, as determined by membrane-specific photoaffinity labelling of protein kinase C [13], indicate an effect of DAG on the lipid–protein kinase C hydrophobic interaction. Protein kinase C contains two functionally distinct domains: a hydrophilic catalytic domain and a hydrophobic phospholipid/phorbol ester/DAG-binding domain [17–20]. Upon tryptic digestion of protein kinase C the two domains can be separated and purified. The part most likely to be affected by hydrophobic interactions is the phospholipid/phorbol ester/DAG-binding fragment, which has been shown to exhibit a higher affinity for PS than does the native enzyme [31]. However, about the exact nature of the hydrophobic interactions between PS and protein kinase C we can only speculate at the moment. The importance of hydrophobic interactions between phospholipids and proteins has been demonstrated for several membrane-bound phospholipid-regulated proteins: for example, the transport of apocytochrome c through lipid bilayers [28] and the activity of the sucrose transporter in human erythrocytes [32].

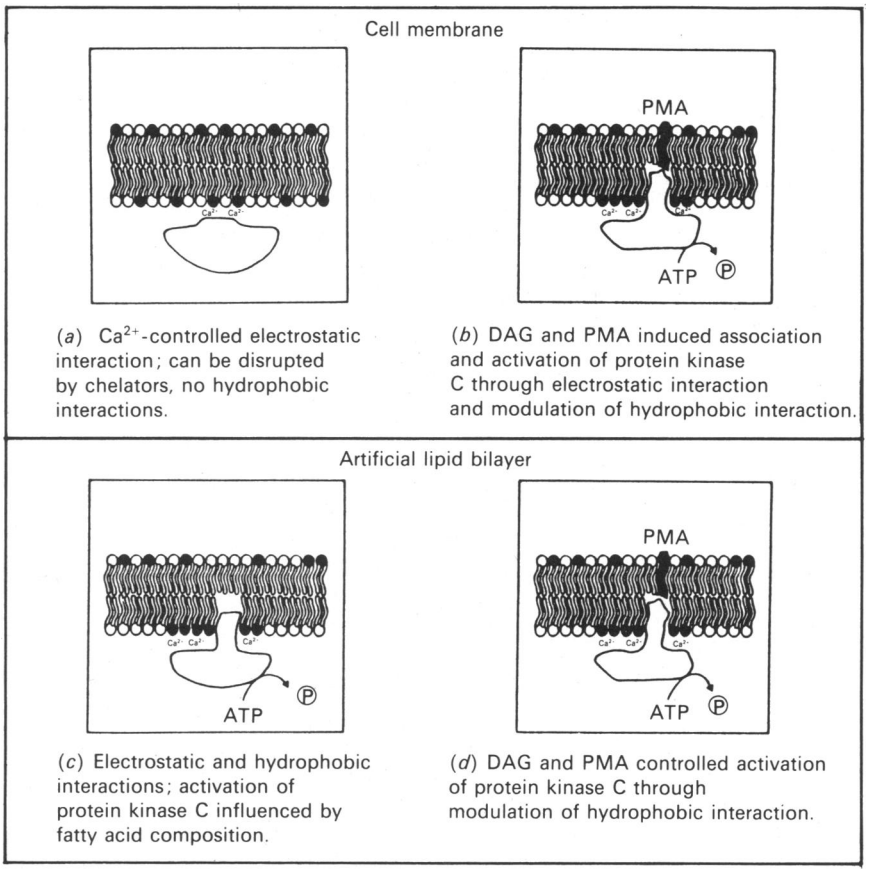

Fig. 6. Schematic model of the activation of protein kinase C in plasma membranes (*a*, *b*) and in artificial bilayers (*c*, *d*), in the absence (*a*, *c*) and the presence (*b*, *d*) of cofactors

Ⓟ is a phosphate molecule transferred from ATP to a substrate molecule by the activated protein kinase C.

Bell and co-workers [33,34] have proposed a model for protein kinase C–PS–DAG–Ca^{2+} interaction based on experiments with mixed micelles of phospholipid to activate protein kinase C; in this model the direct (ionic) interaction between PS molecules and protein kinase C activates the enzyme by inducing a conformational change in the enzyme. In our experiments we have used unilamellar phospholipid bilayers as an alternative approach to imitate physiological conditions. On the basis of these results, we propose an alternative model for the activation of protein kinase C involving hydrophobic interactions, as represented in Fig. 6. From our experiments we conclude that, in non-stimulated cells, protein kinase C is loosely associated with the plasma membrane. This association is dependent on Ca^{2+} and can be disrupted by Ca^{2+} chelators. It most probably is an electrostatic interaction (Fig. 6*a*). When cofactors such as DAG and PMA are added to cells, protein kinase C becomes tightly associated with the plasma membrane, resulting in an active state. This tight association probably involves hydrophobic interaction, since only detergents can dissociate protein kinase C from the plasma membrane under these conditions (Fig. 6*b*). Obviously the precise nature of these hydrophobic interactions is difficult to study in intact cells. For that reason we have analysed in more detail these hydrophobic interactions in reconstitution experiments *in vitro*, under conditions in which Ca^{2+} is not a limiting factor for the activation of the enzyme. Our reconstitution experiments make clear that the activation of protein kinase C by its interaction with phospholipid bilayers is of a more complex nature than that observed by studying protein kinase C–mixed-micelle interaction. As with micelles, the interaction with bilayers requires the presence of Ca^{2+} and negatively charged phospholipids, such as PS. This interaction is, however, not a simple electrostatic one. It cannot be disrupted by Ca^{2+} chelators; detergents are required to separate the enzyme from the phospholipid bilayer, independently of the presence of DAG or PMA, indicating that it is not only an effect of an increased affinity for Ca^{2+}. Furthermore, in this situation the extent of protein kinase C activation is dependent on the fatty acid composition of the activating phospholipid, such that with increasing unsaturation of PS the maximal activity decreases and the dissociation constant for the protein kinase C–PS complex increases (Figs. 3 and 4). This demonstrates that the bilayer–protein kinase C interaction is also hydrophobic in nature and that this hydrophobic interaction is regulatory in the activation of the enzyme (Fig. 6*c*). It should be noted that this effect of fatty acid composition is not due to variations in the amount of bilayer-bound protein kinase C (Fig. 5). Furthermore, no evidence for co-operativity was found for the PS–protein kinase C activation, as the Hill constant was approximately equal to 1 under all conditions, this result being in contrast with the protein kinase C–micelle interaction [33,34].

Under the experimental conditions used here, the

presence of cofactors such as DAG and PMA is not essential for obtaining a maximal activation of protein kinase C. These cofactors decrease the dissociation constant of the PS–protein kinase C complex, independent of the degree of PS saturation. Their effect on the maximal activation of protein kinase C is, however, dependent on the fatty acid composition of the activating phospholipid. With highly saturated PS species, protein kinase C is optimally activated and neither DAG nor PMA is able to stimulate this activity further. When protein kinase C interacts with more unsaturated PS species, these cofactors enhance its activity increasingly with decreasing saturation (Figs. 3 and 4). The simplest explanation for these observations is that DAG and PMA exert their stimulatory effect on the interaction of protein kinase C by a modulation of the hydrophobic interaction between the enzyme and the activating phospholipid (Fig. 6*d*). In this context it is noteworthy that other studies have shown that the acyl chain length and unsaturation of DAG also affect the protein kinase C activation [17,20,35]. Ca^{2+} ions play an important role in the lipid-association and activation process of protein kinase C. In an ideal system *in vitro*, the concentration of free Ca^{2+} should be physiological (1 μM). Since we were investigating hydrophobic interactions, we have consistently added a superoptimal concentration (0.5 mM) of $CaCl_2$ to an assay mixture composed of compounds that affect the free Ca^{2+} concentration (Mg^{2+} ions, EGTA/EDTA, charged lipids etc.). Using a Ca^{2+}-selective electrode (Radiometer T2002) we measured a free Ca^{2+} concentration of 0.19 mM in the absence of protein kinase C. This concentration is above physiological, but increasing or decreasing the Ca^{2+} concentration by a factor 4 did not affect the results described.

Our present results do not indicate the presence of lateral phase separation in the various lipid bilayers. Since the Ca^{2+} concentration and the ratio PS/PC are the factors determining the presence of phase separation and any shift [36], it is of interest to investigate a possible correlation between lipid-dependent activation of protein kinase C and the phase transition or separation in the lipid bilayer.

The PS preparations used in this study were all purified from bovine organs, indicating a non-homogeneous population of lipid molecules. With these preparations little can be said about the exact composition of the PS molecules and a possible preference of protein kinase C for certain PS molecules. However, another phospholipid that does activate protein kinase C, phosphatidylglycerol, can be synthesized with a well defined fatty acid composition. In preliminary experiments with phosphatidylglycerol varying from very unsaturated (di-$C_{18:1}$) to very saturated (di-$C_{16:0}$) we have found comparable results.

The results of the experiments *in vitro* described in this paper open the possibility that the fatty acid composition of membrane phospholipids is one of the regulatory factors in the activation mechanism of protein kinase C *in vivo*.

Thus variation in the hydrophobic part of the plasma membrane could influence the effects of tumour promoters in various cell and tissue types and induce the diversity in cellular responses to stimulation of protein kinase C by tumour-promoting phorbol esters, i.e. inhibition/stimulation or induction of cellular differentiation [37], stimulation or inhibition of cellular growth [38] and sensitivity to induction of tumour promotion [39]. The results demonstrate the need to consider, besides structural features of protein kinase C itself [40], all aspects of bilayer formation in developing an understanding of the Ca^{2+}–phospholipid-dependent activation mechanism of protein kinase C.

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