Conformation of the C1 phorbol-ester-binding domain participates in the activating conformational change of protein kinase C

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The fluorescent phorbol ester 12-*N*-methylanthraniloylphorbol 13-acetate [sapintoxin D (SAPD)] was used as both the activator and the probe for the activating conformational change of the C1 domain of recombinant protein kinase C (PKC) α . Fluorescence emission spectra and steady-state anisotropy measurements of SAPD in fully active membrane-associated PKC show that there is a relatively hydrophobic environment and restricted motional freedom characterizing the phorbol-ester-binding site. SAPD also interacts with the membrane lipids so that it was necessary to resort to time-resolved anisotropy measurements to resolve the signals corresponding to PKC-bound SAPD from that associated with buffer and lipid. In the presence of membrane lipids (unilamellar vesicles of phosphatidylcholine and phosphatidylserine, 4:1 molar ratio) and Ca²⁺, at a concentration sufficient to activate the enzyme fully, a long correlation time characteristic of highly restricted motion was observed for PKC-associated SAPD. The fraction of SAPD molecules displaying this restricted motion, in comparison with the total SAPD including that in lipids and in buffer, increased with increasing concentrations of Ca²⁺ and paralleled the appearance of enzyme activity, whereas the rotational correlation time remained con-

stant. This could be rationalized as an increase in the number of active PKC conformers in the total population of PKC molecules. It therefore seems that there is a distinct conformation of the C1 activator-binding domain associated with the active form of PKC. The addition of SAPD and dioleoyl-*sn*-glycerol together produced an activity higher than that achievable by either activator alone both at concentrations that alone induced maximal activity for the respective activator; this higher activity was associated with a further restriction in SAPD motion. Increasing the cholesterol concentration, the phosphatidylethanolamine concentration, the *sn*-2 unsaturation in phosphatidylcholine and the vesicle curvature each also elevated SAPD-induced PKC activity and again increased the PKC-associated SAPD rotational correlation time. In summary, the rotational correlation time of PKC-bound SAPD, extractable from a single time-resolved fluorescence anisotropy measurement, provides a novel probe for the involvement of interactions between the C1 domain and phorbol ester in the modulation of PKC activity.

Key words: sapintoxin, time-resolved fluorescence.

INTRODUCTION

The protein kinase C (PKC) α group of enzymes are key regulators of numerous signalling pathways (reviewed in [1–3]). The PKC family consists of Ca²⁺-dependent conventional (cPKC α , β _{I/II} and γ), Ca²⁺-independent or non-conventional (nPKC δ , ϵ , θ , η and μ) and Ca²⁺- and dioleoyl-*sn*-glycerol (DAG)-independent or atypical (aPKC ζ , ι and λ) isoenzymes [4]. The cPKC isoforms translocate from the cytosol to a membrane surface owing to an increase in intracellular Ca²⁺ and DAG concentrations, commonly produced by G-protein-coupled receptor-induced activation of phospholipase C. Interaction of DAG or phorbol esters with the C1 domain, in the presence of Ca²⁺, permits an activating conformational change in the enzyme that consists of an unfolding of the enzyme and exposure of the substrate-binding site.

The activator-binding site of PKC resides within the regulatory region in the C1 domain and consists of two cysteine-rich zinc-finger-like motifs, C1A and C1B [5]. The basic structure consists of the pattern HX₁₂CX₂CX_{10–14}CX₂CX₄HX₂CX₇C (single-letter codes) [6]. There have been several structural studies on isolated C1 domains [7–10]. The structure of the C1B domain of PKC δ has been determined by X-ray diffraction, showing the position of a phorbol ester within its binding site [8]. Other structural studies that have been performed include an NMR solution-state structural investigation of both the C1A and C1B domains of PKC γ [9] and the C1B domain of PKC α [7] and PKC γ [13]. By

using molecular modelling and site-directed mutagenesis of the phorbol-ester-binding domain, essential features of the phorbol ester structure required to interact optimally with the binding region have been determined [10]. It was shown that the C-20 and C-3 positions in the phorbol ester structure have important contributions to the overall binding energy. Although two histidine residues and six conserved cysteine residues seem to be the basic minimum requirement for the binding of phorbol ester [14], a number of other amino acid residues have an important role and are highly conserved [10,15], whereas the C-terminal region did not seem to be important in directing phorbol ester binding.

Currently it is generally assumed that the interaction of an activator with the C1 domain serves to facilitate the association of PKC with the membrane but that it is not involved in the activating conformational change. Thus the interaction of phorbol esters with the C1 domain seems to cap a hydrophobic region and this might facilitate the interaction of PKC with the membrane [8]. Although an activator has to bind to the C1 domain of PKC to allow the activating conformational change in the enzyme, it is not known whether the conformation of the C1 domain itself also changes on activation, although one study reported that interaction of the C1B domain of PKC γ with lipids resulted in a number of amino acid residues' exhibiting NMR line broadening [13]. If it were to change on activation, this would imply that the interaction of activator with the C1 domain

Abbreviations used: DAG, dioleoyl-*sn*-glycerol; PtdSer, phosphatidylserine; NBD-PtdSer, *N*-(7-nitro-2-1,3-benzoxadiazole-4-yl)-PtdSer; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; PKC, protein kinase C; POPC, 1-palmitoyl-2-oleoyl-PtdCho; SAPD, sapintoxin D (12-*N*-methylanthraniloylphorbol 13-acetate).

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Figure 1 Structure of PMA (a) and SAPD (b)

of PKC could also have a role in setting the level of activity and possibly the proteolytic sensitivity, which could potentially be exploited in the control of PKC involvement in signalling pathways. To address this question in the present study, the conformational change of the C1 activator-binding domain was probed by using time-resolved fluorescence spectroscopic measurements of the fluorescent phorbol ester 12-(*N*-methylantraniloyl)phorbol 13-acetate [sapintoxin D (SAPD)] to determine whether this domain participates in the activating conformational change of the enzyme.

The naturally occurring phorbol esters SAPD (Figure 1b) and sapintoxin A (12-*N*-methylantraniloyl-4-deoxyphorbol 13-acetate [16,17]) are fluorescent owing to the methylantranilate group; like other phorbol esters they activate PKC potently [18–20]. In the present study the fluorescent properties of SAPD, in terms of emission spectra and steady-state and time-resolved anisotropy, were used to probe the hydrophobic properties and conformational change in the PKC C1-domain activator-binding pocket on the activation of PKC. The dynamic properties of SAPD bound to PKC were used to reveal conformational changes in the domain in response to association with membrane, Ca^{2+} -induced activation, the addition of phorbol ester and DAG together, and altered properties of the lipid bilayer. The last of these was investigated with lipid vesicles with increased levels of cholesterol, phosphatidylethanolamine (PtdEtn), phosphatidylcholine (PtdCho) unsaturation and vesicle curvature, all of which enhanced enzyme activity. The results are consistent with the motional freedom of the phorbol ester within the C1 domain reflecting the state of activation of the enzyme.

EXPERIMENTAL

Materials

All the phospholipids, including *N*-(7-nitro-2-1,3-benzoxadiazole-4-yl)-phosphatidylserine (NBD-PtdSer), were purchased from Avanti Polar Lipids (Alabaster, AL, U.S.A.). The lipid concentrations were determined by phosphorus assay [21]. SAPD was from LC Laboratories (Woburn, MA, U.S.A.). PMA (Figure 1a) was from Sigma (St. Louis, MO, U.S.A.). ATP was from Boehringer Mannheim (Indianapolis, IN, U.S.A.) and [γ - ^{32}P]ATP was from DuPont NEN (Boston, MA, U.S.A.). A peptide substrate (myelin basic protein, MBP_{4–14}) was custom-synthesized by the Protein Chemistry Facility of the Jefferson Cancer Institute, Thomas Jefferson University, PA, U.S.A. Dioxan and other solvents used in this study were of analytical grade and were obtained from Fisher Scientific (Pittsburgh, PA, U.S.A.).

Preparation of PKC α

PKC α was prepared with the baculovirus-Sf9 (*Spodoptera frugiperda*) insect cell expression system [22], and purified as described previously [23]. Purified PKC α displayed a single band on a silver-stained electrophoresis gel. Assays of PKC α activity followed a previously described method that involved the measurement of the initial rate of incorporation of ^{32}P into the peptide substrate myelin basic protein_{4–14} [23].

Phospholipid vesicles

Large unilamellar vesicles were prepared as described previously [24]. In brief, aliquots of the required phospholipids in chloroform were placed in a test tube and the solvent was removed under a stream of nitrogen. Buffer [50 mM Tris/HCl (pH 7.4)/0.1 mM EGTA] was then added to give a total lipid concentration of 1 mM. After vortex-mixing, the sample was passed through a polycarbonate filter (100 nm pore size) with the Avestin Liposofast Extruder (Avestin, Ottawa, Canada). Large unilamellar vesicles consisting of PtdCho and brain phosphatidylserine (PtdSer) at a 4:1 molar ratio, with or without DAG, cholesterol or 1-palmitoyl-2-oleoyl-PtdEtn, as indicated, were diluted to a final lipid concentration of 100 μM for time-resolved measurements or 50 μM for steady-state spectra and anisotropy measurements. All the measurements were performed at 30 °C.

Fluorescence spectroscopy

Fluorescence data were collected with an SLM 48000 multi-frequency phase-modulation fluorimeter. Steady-state spectra and anisotropy were measured with a Xenon lamp as the excitation source at 360 nm for SAPD or at 470 nm for NBD-PtdSer. Time-resolved fluorescence measurements used a Liconix HeCd laser (model 4240NB, 325 nm) excitation source, modulated sinusoidally by a radio frequency from 5 to 150 MHz. SAPD emission was collected through a 385 nm redpass filter and a Glan–Thompson polarizer set at the desired orientation for anisotropy measurements. For measurements of steady-state fluorescence spectra, the emissions were collected through a Glan–Thompson polarizer set at the magic angle and a single monochromator. For both steady-state and time-resolved anisotropy measurements, a T-format configuration was used. The principle and operational details are as published elsewhere [25,26]. NBD-PtdSer emission was collected through 520 nm redpass filters.

For lifetime measurements, an aqueous solution of rabbit liver glycogen was used as a light scattering reference. The experimental errors used in fluorescence lifetime data analysis were the averaged errors over the range of frequencies collected in data acquisition, usually approx. 0.2 ° in the phase and approx. 0.002 in the modulation. For time-resolved fluorescence anisotropy measurements, the averaged experimental errors were usually 0.3 ° in the phase and 0.003 in the modulation.

Data analysis

Steady-state anisotropy r_s was calculated from the equation:

$$r_s = (I_{VV} - GI_{VH}) / (I_{VV} + 2GI_{VH}) \quad (1)$$

where G is the instrumental correction factor, $G = I_{HV}/I_{HH}$, I_{VV} is the emission intensity with vertically polarized excitation and emission and I_{VH} is the emission intensity with vertically polarized excitation and horizontally polarized emission. Phase and modulation data were analysed with the Globals Unlimited software

(Laboratory of Fluorescence Dynamics, University of Illinois, Urbana-Champaign, IL, U.S.A.) [27]. The data were fitted to several analytical models and compared on the basis of χ^2_R and residuals. The analyses of the fluorescence lifetime and time-resolved anisotropy followed previously published procedures [28], with exponential decays for lifetime and isotropic rotator models for SAPD in buffer, bilayers and bound to PKC as follows:

$$r(t) = r_0[f_1\exp(-t/\phi_1) + f_2\exp(-t/\phi_2)] \quad (2)$$

or

$$r(t) = r_0[f_1\exp(-t/\phi_1) + f_2\exp(-t/\phi_2) + f_3\exp(-t/\phi_3)] \quad (3)$$

where r_0 is the limiting anisotropy, ϕ_1 , ϕ_2 and ϕ_3 are the rotational correlation times, f_1 , f_2 and f_3 are the fractional intensities associated with the respective rotational correlation times and τ is the fluorescence lifetime.

RESULTS

Steady-state emission spectra and anisotropy of SAPD

The excitation and emission spectra of SAPD in solvents and in membrane lipid vesicles are shown in Figures 2 and 3. SAPD is soluble in both buffer and hexane but has an approx. 40 nm lower emission maximum in the latter (Figure 2a). This is shown

Figure 2 Fluorescence emission of SAPD in solvents

(a) Fluorescence excitation and emission spectra of SAPD in hexane (solid lines) and Tris/HCl buffer (dotted lines). (b) Fluorescence spectra of SAPD in solvents with different dielectric constants with various ratios of dioxan to water (0:100, 10:90, 20:80, 40:60, 60:40, 80:20 and 100:0, all v/v) of known dielectric constants (79, 76, 64, 42, 25, 10 and 2.0 [50]). The excitation wavelength was set at 355 nm. Other details are as described in the Experimental section.

Figure 3 Fluorescence emission of SAPD in vesicles: effect of addition of PKC α

The spectra were deconvoluted with Peakfit (Jandel Scientific) into individual peaks corresponding to SAPD in buffer (approx. 435 nm) and in hexane (approx. 395 nm). The intensity of the blue-shifted peak was greater after the addition of PKC α (10 μ g/ml) and increased further with increased PKC α concentration; the position of the emission maximum was unchanged. The excitation wavelength was set at 355 nm. Other details are as described in the Experimental section. Symbols: $\bullet$, in buffer only; $\blacktriangle$, with vesicles (deconvoluted peaks are shown as broken and dot-dashed lines); $\blacksquare$, with vesicles and PKC α (deconvoluted peaks are shown as dotted and solid lines).

more clearly in Figure 2(b), which indicates that the emission maximum moves to lower wavelengths with decreasing dielectric constant of the solvent. This suggests that SAPD is a useful probe of the dielectric constant or 'hydrophobicity' of the phorbol-ester-binding site in the C1 domain of PKC. Accordingly, emission spectra were recorded for SAPD in 1-palmitoyl-2-oleoyl-PtdCho (POPC)/brain PtdSer vesicles with and without PKC α and Ca $^{2+}$ (100 μ M). The spectra (Figure 3) show the presence of two components for SAPD in lipid vesicles. One component, centred at approx. 435 nm, is due to buffer-associated SAPD and the other, at approx. 395 nm, is due to membrane lipid-bilayer-associated SAPD; the relative intensity of this shorter-wavelength component increased in the presence of PKC and Ca $^{2+}$.

To explore further the phorbol-ester-binding pocket in PKC, the steady-state anisotropy (r_s) of SAPD was determined in membrane lipids, before and after the addition of PKC and Ca $^{2+}$, as shown in Figure 4(a). The fact that SAPD interacts with the lipids is confirmed by the fairly high anisotropy value of approx. 0.09, indicative of a restricted environment. On the addition of PKC α (without Ca $^{2+}$), the anisotropy increased as a result of the combination of both membrane-associated and PKC-associated SAPD signals. A further increase was observed on the addition of Ca $^{2+}$. This indicated that the motional freedom of SAPD decreased within the phorbol-ester-binding pocket of the C1 domain of PKC when the enzyme became associated with the lipid bilayer. This is consistent with the finding that phorbol

Figure 4 Steady-state fluorescence anisotropy (r_s) of SAPD

(a) Effect of Ca $^{2+}$ and PMA (TPA), showing that the latter displaced SAPD from PKC α (10 μ g/ml). In the absence of PKC α , the anisotropy of SAPD remained unchanged on the addition of PMA and Ca $^{2+}$. (b) The biologically inactive phorbol ester 4 α -PMA (4 α TPA) was unable to displace high-affinity SAPD. Concentrations: Ca $^{2+}$, 0.1 mM; SAPD, 1 μ M; PMA, 1 μ M; 4 α -PMA, 1 μ M; vesicles, 50 μ M POPC/brain PtdSer (4:1, mol/mol). Other details are as described in the Experimental section.

esters have an increased affinity for membrane-associated compared with non-membrane-associated PKC. For example, the K_d for phorbol 12,13-dibutyrate for the free C1B region of PKC δ is 157 nM, in contrast with 1.9 nM in the presence of lipids [15]. Importantly, the addition of PMA decreased the SAPD anisotropy, indicating that the increased SAPD anisotropy was due to a specific SAPD–PKC interaction. In agreement with this, 4 α -PMA, the inactive epimer of PMA, did not compete for SAPD, as shown in Figure 4(b), and neither Ca $^{2+}$ nor PMA had any effect on the anisotropy of membrane-lipid-associated SAPD in the absence of PKC. In contrast, bryostatin, which also activates PKC by interaction at the phorbol-ester-binding site, also competed for SAPD and decreased the anisotropy (results not shown).

Time-resolved fluorescence anisotropy of SAPD

The observed steady-state anisotropy of SAPD, in the presence of membrane lipids and PKC, is a combination of the signals from buffer, membrane lipids and PKC-associated SAPD. This makes it difficult to separate effects on phorbol-ester–PKC interactions from the motional properties of SAPD in the membrane lipids. SAPD fluorescence (in terms of intensity as shown in the spectra) increases on the addition of PKC; one of the factors, besides a more hydrophobic environment, would be the additional SAPD-binding sites provided by PKC to that provided by the lipids. For this reason, fluorescence intensity is

Table 1 Analysis of time-resolved fluorescence data of SAPD on interaction with PKC α : fluorescence lifetimes

τ_1 , τ_2 , fluorescence lifetimes; f_1 , f_2 , fractional intensities; χ^2_R , reduced χ^2 (for one representative data set). Concentrations: lipid vesicles, 100 μ M; SAPD, 1 μ M; Ca $^{2+}$, 0.1 mM; PKC α and glutathione S-transferase-bound C1 domain, 20 μ g in 50 mM Tris/HCl, pH 7.4 at 30 °C. Other details are as described in the Experimental section. Results are means $\pm$ S.D.

Condition	τ_1 (ns)	f_1	τ_2 (ns)	f_2	χ^2
Buffer	1 τ 5.05 $\pm$ 0.08	1.00			30.49
	2 τ 5.93 $\pm$ 0.16	0.90 $\pm$ 0.03	2.72 $\pm$ 0.09	0.10 $\pm$ 0.03	1.65
Vesicles	1 τ 5.93 $\pm$ 0.22	1.00			74.99
	2 τ 7.27 $\pm$ 0.26	0.86 $\pm$ 0.04	2.52 $\pm$ 0.55	0.14 $\pm$ 0.04	1.04
PKC α	1 τ 5.60 $\pm$ 0.12	1.00			253
	2 τ 7.06 $\pm$ 0.17	0.80 $\pm$ 0.02	2.40 $\pm$ 0.28	0.20 $\pm$ 0.02	0.96
Ca $^{2+}$	1 τ 5.67 $\pm$ 0.12	1.00			189
	2 τ 7.23 $\pm$ 0.07	0.83 $\pm$ 0.01	2.36 $\pm$ 0.28	0.17 $\pm$ 0.01	1.87

less useful for quantifying the distribution of SAPD between different environments. The fluorescence lifetimes and steady-state anisotropies for lipid-bound and PKC-bound SAPD could also not be easily resolved. In contrast, it was found that SAPD bound to PKC displayed markedly different rotational correlation times from that for free and lipid-bound SAPD, also the fractional contributions available from the analysis provide information on the extent of membrane association.

To probe the PKC–SAPD interaction specifically, the time-resolved anisotropy of SAPD was measured, permitting the separation of lipid-bound SAPD from PKC-bound SAPD. These measurements also require the fluorescence lifetime for the calculation (see Tables 1–3). The phase and modulation data were then analysed in the form of one-, two- or three-rotational correlation times as described in the Experimental section. The longer the rotational correlation time becomes, the more restricted is the motional freedom of a fluorophore. For PKC without lipids or Ca $^{2+}$, SAPD has both a fast (buffer-associated SAPD) and a slower rotational correlation time of approx. 8 ns. A correlation time of approx. 8 ns was also observed for a C1 domain bound to glutathione S-transferase, indicating that this represents PKC-bound SAPD (see Tables 1–3). For lipid vesicles alone, apart from the signal for SAPD in buffer, an approx. 10 ns rotational correlation time was found, which was consistent with SAPD's residing in the lipid headgroup region. For PKC with vesicles and 100 μ M Ca $^{2+}$, a two-rotational correlation time analysis again revealed a fast correlation time for unbound SAPD, whereas a second correlation time of 16 ns was obtained. This value represents two underlying SAPD populations, one being lipid-associated and the other PKC-associated. Therefore, to account properly for the total of three populations in the sample, a three-correlation time analysis was employed and a long correlation time of approx. 42 ns was obtained, which was consistent with SAPD's being strongly bound within the C1 domain. Although the χ^2_R for the three-correlation time analysis was not improved over that for a two-correlation time analysis, this could be attributed to the size of the errors as shown in a simulation in Table 2, using the simulation feature of the Global software. Only at errors of less than 0.1 ° and less than 0.001 in the phase and modulation respectively was the χ^2_R lower for a three-correlation time analysis of artificial data with added errors after reanalysis. This level of error is not achievable with current instrumentation. In conclusion, for the three-correlation time

Table 2 Analysis of time-resolved fluorescence data of SAPD on interaction with PKC α : time-resolved anisotropy simulations

The following rotational correlation times and fractions were fed into the Global analysis software and the phase and modulation values were determined with artificial 'error' values. The data were then analysed as two- and three-rotational correlation times to determine the values of χ^2_R for various errors.

Error	Input values...	ϕ_1 (ns)	f_1	ϕ_2 (ns)	f_2	ϕ_3 (ns)	f_3	χ^2
		60	0.15	10	0.15	0.2	7	
0.2/0.02	2 ϕ	21.85	0.33	0.27	0.67			0.63
	3 ϕ	61.50	0.15	12.62	0.14	0.20	0.71	0.51
0.3/0.003	2 ϕ	21.86	0.32	0.26	0.68			1.22
	3 ϕ	69.45	0.11	11.50	0.17	0.18	0.72	1.14
0.1/0.001	2 ϕ	20.93	0.29	0.21	0.71			1.38
	3 ϕ	68.01	0.14	10.60	0.16	0.20	0.70	0.45

Table 3 Analysis of time-resolved fluorescence data of SAPD on interaction with PKC α : time-resolved anisotropy data

The rotational correlation times ϕ_1 , ϕ_2 and ϕ_3 and the associated fractions f_1 , f_2 and f_3 from analysis of the phase and modulation data (average errors 0.3 and 0.003 respectively). The superscripts P, L and B refer to which of PKC (P), lipid (L) or buffer (B) that the respective correlation time and fractions is considered to be associated with. Bold text indicates which of a one-, two- or three-rotational correlation time analysis is considered the most appropriate for the given experimental condition. χ^2_R reduced χ^2_R for a representative data set. Abbreviation: GST-C1, glutathione S-transferase bound to C1 domain. Results are means $\pm$ S.D.

Condition	ϕ_1 (ns)	f_1	ϕ_2 (ns)	f_2	ϕ_3 (ns)	f_3	χ^2_R
Buffer	1ϕ 0.15^B $\pm$ 0.01	1.00					3.09
Vesicles	1 ϕ 3.43 $\pm$ 0.56	1.00					45.16
	2ϕ 11.39^L $\pm$ 0.76	0.28^L $\pm$ 0.06	0.29^B $\pm$ 0.08	0.72^B $\pm$ 0.06			0.89
	3 ϕ 0.19 $\pm$ 0.01	0.78 $\pm$ 0.02	9.69 $\pm$ 2.04	0.21 $\pm$ 0.15	10.81 $\pm$ 0.65	0.01 $\pm$ 0.13	1.18
GST-C1 (buffer only)	1 ϕ 5.17	1.00					15.41
	2ϕ 7.92^P	0.36^P	0.22^B	0.64^B			0.46
	3 ϕ 0.19	0.68	8.33	0.26	7.85	0.06	0.69
PKC α (buffer only)	1 ϕ 3.26	1.00					23.56
	2ϕ 7.99^P	0.15^P	0.19^B	0.85^B			2.01
	3 ϕ 0.20	0.79	8.79	0.10	7.09	0.11	2.14
Vesicles + PKC α + 100 μ M Ca ²⁺	2 ϕ 16.58 $\pm$ 0.94	0.32 $\pm$ 0.09	0.27 $\pm$ 0.15	0.68 $\pm$ 0.09			1.16
	3ϕ 42.58^P $\pm$ 2.94	0.15^P $\pm$ 0.05	8.58^L $\pm$ 0.98	0.12^L $\pm$ 0.05	0.22^B $\pm$ 0.12	0.73^B $\pm$ 0.05	1.07
Vesicles + PKC α + 1 μ M Ca ²⁺	3ϕ 43.05^P	0.07^P	9.22^L	0.15^L	0.19^B	0.78^B	2.75
Vesicles + PKC α + 100 μ M Ca ²⁺ + DAG	3ϕ 69.35^P	0.09^P	10.82^L	0.09^L	0.19^B	0.82^B	0.65
Vesicles + PKC α + 100 μ M Ca ²⁺ + 3 μ M PMA	2ϕ 10.90^L	0.11^L	0.19^B	0.89^B			3.72
	3 ϕ 0.19	0.89	10.37	0.10	10.05	0.01	3.93
Vesicles + PKC α + 100 μ M Ca ²⁺ + 0.5 μ M bryostatin	2ϕ 13.70^{P/L}	0.13^{P/L}	0.18^B	0.87^B			1.09
	3 ϕ 0.80	0.88	9.84	0.01	10.26	0.11	1.52

analysis it was not possible to use the χ^2_R criterion alone to test whether it was returning physically realistic data. In addition, when SAPD in (1) glutathione S-transferase-bound C1 domain, (2) PKC alone or (3) PKC with lipids, Ca²⁺ and bryostatin was analysed as a three-correlation time model the analysis returned two identical correlation times in addition to the third buffer-associated SAPD species, as shown in Table 3. The latter result with bryostatin can be explained on the basis of a previous observation that high-affinity binding of phorbol ester is displaced by bryostatin [29]. For these three cases, only two species are in fact present and a two-correlation time model is appropriate.

Effect of varying the concentrations of PKC and PMA

To test further the robustness of the model of three SAPD locations (buffer, lipid-bound and PKC-bound), the concentrations of PKC and PMA were varied for fully activated PKC with lipid and Ca²⁺. On increasing the concentration of PKC this

should increase the relative number of SAPD binding sites in PKC in the system, whereas the addition of PMA should displace the SAPD from PKC, increasing or decreasing the fractional contribution of SAPD bound to PKC respectively. In neither case should the value of the rotational correlation time of SAPD bound to PKC be affected, only its fractional contribution. Accordingly, data sets were collected for various concentrations of PMA and PKC. The entire respective data set was then analysed as a single global data set (one for varied PMA and one for PKC) with r_0 , the rotational correlation times for SAPD in buffer and lipid-associated SAPD (determined separately) kept constant and 'linked' in the analysis. The result is summarized in Figure 5, in which it can be seen that the fractional contribution of SAPD bound to PKC did indeed vary (Figure 5a), whereas its rotational correlation time remained constant (Figure 5b). Note that at 3 μ M PMA most of the SAPD had been displaced from PKC, so a three-correlation time analysis was no longer needed and a two-correlation time model for lipid- and buffer-associated SAPD was then appropriate (see Table 3).

Figure 5 Time-resolved fluorescence anisotropy of SAPD: effect of variation of PMA (TPA) or PKC

(a) The fraction of PKC-associated SAPD increased when the PKC α concentration was increased or when that of PMA was decreased. (b) The rotational correlation time of PKC-associated SAPD was unchanged over the range of PMA and PKC concentrations (■, PMA; ●, PKC α). Each point is a single independent observation. Other details are as described in the Experimental section.

Effect of variation of Ca^{2+} on time-resolved fluorescence anisotropy parameters

The effect of increasing the Ca^{2+} concentration from 0 to 100 μM on the fractional contribution of SAPD bound to PKC and its associated long rotational correlation time is shown in Figure 6(a). The first point (□) is for PKC-bound SAPD without lipids or Ca^{2+} , a short correlation time being shown as discussed above. In the presence of lipids the rotational correlation time jumped to 42 ns, showing that the SAPD, as a phorbol ester, induced association with membrane and an altered C1 domain conformation, as reflected in the longer rotational correlation time. In this case the effect occurred independently of Ca^{2+} because the association with membrane was driven by the relatively high level of the SAPD; however, under these conditions most of the PKC was still inactive as reflected by the low fractional contribution of the long correlation time. As the Ca^{2+} concentration was increased further, the fractional contribution of PKC-associated SAPD increased in parallel with the appearance of activity (Figure 6b). This is not explainable as a shift in the equilibrium of free and membrane-bound PKC towards more complete association with membrane. On the contrary, by using the fluorescence anisotropy of NBD-PtdSer to monitor association with membrane it was found that the binding of PKC to membrane was complete at low (micromolar) Ca^{2+} (Figure 6a), as demonstrated elsewhere [30]. Therefore the fraction of the

Figure 6 Effect of increased Ca^{2+} levels on the PKC-bound SAPD rotational correlation time, its fractional contribution, and NBD-PtdSer anisotropy (a), and effect of increased Ca^{2+} levels on PKC α activity (b)

(a) The fraction of PKC-associated SAPD (●) increased (10 $\mu\text{g}/\text{ml}$ PKC) with increasing Ca^{2+} concentration, whereas the rotational correlation time (■) was independent of Ca^{2+} concentration. For comparison, the rotational correlation time for PKC-bound SAPD without lipids or Ca^{2+} is shown (□). Each point is a single independent observation. The anisotropy response of NBD-PtdSer to increased Ca^{2+} (▲) reflects membrane association and does not follow the activity curve (○) in (b). Where error bars are shown they are means $\pm$ S.D. for triplicate determinations; for activities the errors were smaller than the symbols. Other details are as described in the Experimental section.

long correlation time of approx. 40 ns, rather than its value, is correlated with the appearance of activity.

Effect of interaction with DAG

Previously we have shown that the addition of DAG and phorbol ester together produces a level of activity greater than that achievable by either activator alone, both being at concentrations that alone produced maximal stimulation for the respective activator [29]. The activities for DAG, SAPD and SAPD plus DAG were 0.71 ± 0.09 (mean $\pm$ S.D.), 0.82 ± 0.06 and 2.1 ± 0.08 nmol of ATP/min per μg of protein respectively. When a three-correlation time analysis was performed on SAPD with PKC, in the presence of POPC/brain PtdSer vesicles containing 4% (mol/mol) DAG, there was a long correlation time of 69 ns, compared with 43 ns without DAG (Table 3). Thus the elevated activity seems to be related to the increased rotational correlation time of PKC-bound SAPD. In previous studies we have shown, by using PKC tryptophan-to-SAPD resonance energy transfer, that DAG displaces SAPD bound with low affinity but increases the affinity of SAPD bound with high affinity, in keeping with this observation [29].

Figure 7 Effect of cholesterol on the steady-state and time-resolved fluorescence anisotropy of SAPD and on PKC α activity

Effect of increased cholesterol on steady-state anisotropy with (■) and without (●) PKC α (10 μ g/ml) (top panel), rotational correlation times for PKC-bound SAPD (■) and lipid-bound SAPD (●) (middle panel), and the specific activity of PKC α (▲) (bottom panel). Each point is a single independent observation except for activities, which are the means $\pm$ S.D. for three measurements; error bars not shown are smaller than the symbols. Other details are as described in the Experimental section.

Effect of variation of vesicle lipid physical properties

To examine further the relationship between the C1 phorbol-ester-binding domain of SAPD and the activity of PKC, the physical properties of the vesicles were varied in a manner known to modify enzyme activity. Lipid molecules, such as PtdEtn, which display polymorphic properties, can be thought of as being cone-shaped owing to the relatively small volume of the head group compared with that of the acyl chains [31]. This destabilization of the bilayer surface interactions is exacerbated by increased phospholipid unsaturation, by cholesterol [32] and by other hydrophobic or amphiphilic compounds [33] including certain alcohols and anaesthetics [34]. The addition of PtdEtn or cholesterol, increased lipid unsaturation or a smaller vesicle curvature each increases PKC activity [35–41]. Accordingly these parameters were varied systematically and the effects on activity and PKC-associated SAPD rotational dynamics were determined.

The effect of increasing the cholesterol concentration on steady-state and time-resolved fluorescence anisotropy and on activity is shown in Figure 7. Increasing the cholesterol concentration decreased the steady-state anisotropy of SAPD in lipid vesicles in the absence of PKC. However, in the presence of PKC α and Ca²⁺ the steady-state anisotropy increased slightly.

Figure 8 Effect of PtdEtn on the steady-state and time-resolved fluorescence anisotropy of SAPD and on PKC α activity

Effect of increased PtdEtn (at the expense of POPC in the POPC/brain PtdSer vesicles) on steady-state anisotropy with (■) and without (●) PKC α (10 μ g/ml) (a), on rotational correlation times for PKC-bound SAPD (■) and lipid-bound SAPD (●) (b) and on the specific activity of PKC α (▲) (c). Each point is a single independent observation except for activities, which are the means $\pm$ S.D. for three measurements; error bars not shown are smaller than the symbols. Other details are as described in the Experimental section. Abbreviation: POPE, 1-palmitoyl-2-oleoyl-PtdEtn.

Clearly, because both SAPD in the lipids and SAPD associated with PKC were contributing to the signal, and both were likely to change, it was not possible to extract information on SAPD–PKC interactions from this measurement alone.

The rotational correlation times for SAPD in lipid vesicles with various cholesterol concentrations were first determined (without PKC); the value decreased with increasing cholesterol concentration (Figure 7). The results with PKC present were then analysed by fixing the respective lipid- and buffer-associated SAPD rotational correlation times; a global analysis was then performed over all cholesterol levels, the long rotational correlation time being left free. The values returned are shown in Figure 7 (middle panel): they increased from 40 ns to almost 100 ns with increasing cholesterol concentration, in parallel with an increasing enzyme activity.

The effects of increased PtdEtn concentration (Figure 8) and unsaturation in PtdCho (Figure 9) on PKC activity and fluorescence anisotropy showed qualitatively similar results to those obtained for cholesterol. Again, the long rotational correlation time for PKC-associated SAPD increased, with a parallel increase in activity being obtained. For varied unsaturation in

Figure 9 Effect of increased *sn*-2 PtdCho unsaturation on steady-state and time-resolved fluorescence anisotropy of SAPD and PKC α activity

Effect of increased *sn*-2 PtdCho *cis*-unsaturation in terms of one (POPC), two (1-palmitoyl-2-linoleoyl-PtdCho), four (1-palmitoyl-2-arachidonoyl-PtdCho) and six (1-palmitoyl-2-docosa-hexaenoyl-PtdCho) double bonds per PtdCho in PtdCho/brain-PtdSer (4:1, mol/mol) vesicles on steady-state anisotropy with (■) and without (●) PKC α (10 μ g/ml) (a), on rotational correlation times for PKC-bound SAPD (ϕ_3 , ■) and lipid-bound SAPD (ϕ_2 , ●) (b) and on the specific activity of PKC α (▲) (c). Each point is a single independent observation except for activities, which are the means $\pm$ S.D. for three measurements; error bars not shown are smaller than the symbols. Other details are as described in the Experimental section.

Table 4 Effect of vesicle size on rotational motion of SAPD on interaction with PKC α

The size of large unilamellar vesicles [POPC/brain-PtdSer (4:1 mol/mol)] was regulated by the use of 30 and 100 nm pore-size polycarbonate filters. Other details are as described in the Experimental section.

Vesicle size (nm)	f_1	ϕ_1 (ns)
100	0.16	42.67
30	0.09	75.17

PtdCho, *sn*-2 unsaturation was increased by using vesicles in which the PtdCho component had oleoyl ($n=9$), linoleoyl ($n=6$), arachidonoyl ($n=6$) and docosahexaenoyl ($n=3$) fatty acyl chains in the *sn*-2 position (one, two, four and six double bonds per PtdCho molecule respectively) and palmitoyl in the *sn*-1 position. Finally, the effects of two different lipid vesicle sizes were compared, as shown in Table 4. It can be seen that the smaller

vesicles induced a higher level of PKC activity, as observed previously [38]; again, this was found to be associated with an increase in the PKC-associated SAPD rotational correlation time.

DISCUSSION

In the present study the motional properties of a phorbol ester bound to PKC were determined for the first time. The *N*-methylanthraniloyl fluorescent moiety of SAPD is attached to the C-12 position on the phorbol ester ring and probes the hydrophobic region normally taken up by the acyl chain attached to the C-12 position. Previous studies have shown that the presence and properties of the acyl chains attached at the C-12 position regulate association with the membrane and insertion of PKC into the membrane [42]. The results of the present study show that the conformation of this region of the phorbol-ester-binding pocket responds in a specific manner to factors that modulate the activity of the enzyme.

From measurements of emission spectra and steady-state fluorescence anisotropy, three distinguishable environments for SAPD were observed. Initially SAPD binds specifically to PKC. This is shown by its ability to activate PKC with a high potency, similar to that of PMA [29], and the finding that PMA displaces SAPD from PKC, as shown by the decrease in SAPD steady-state anisotropy by PMA, which is indicative of specific binding. The higher steady-state anisotropy for SAPD with fully activated membrane-associated PKC, compared with that for SAPD in lipids alone, suggests that the SAPD bound in the C1 region is in a more restricted motional environment than that located in the membrane lipids. For PKC (without membrane lipids) the rotational correlation time of SAPD is 8 ns, similar to the value for the isolated C1 domain. Apart from PKC, SAPD also associates with membrane lipids, probably in the headgroup region, in which it displays a rotational correlation time of 10 ns, and lastly a fast-rotating species for SAPD in buffer is found.

Overall there are several observations supporting the argument that the distinct long correlation time of SAPD is associated with active (membrane-associated) PKC-associated SAPD. First, either for SAPD with PKC alone or for SAPD in membranes lipids without PKC, a long rotational correlation time is never produced. Secondly, there is a remarkably high degree of internal self-consistency in the results that strongly support this interpretation of the results. For example, increased levels of PMA decrease the proportion (fractional contribution) of SAPD displaying the long correlation time but not its value (i.e. PMA displaces the SAPD from PKC but not from the membrane), whereas increasing the level of PKC provides more SAPD sites and the fraction, but not the correlation time, increases as expected. Increased cholesterol concentrations, PtdEtn concentrations and unsaturation in PtdCho all increase the activity of PKC and the PKC-SAPD rotational correlation time but have a marginal effect on the fraction. Thirdly, the model is consistent with the earlier finding that the affinity of phorbol esters for PKC increases markedly on association with membrane [43].

It is important to determine whether the longer correlation time for PKC-bound SAPD arises from the fluorophore's sensing an altered C1-domain activator-binding pocket conformation or a different position of the fluorophore in the bilayer. The latter would occur if the SAPD methylanthraniloyl fluorophore were to reside at the C1-lipid interface, where it might sense the motional environment in the bulk lipid. In that case, if there were a tighter PKC-membrane binding associated with the fully active PKC conformer, this might induce a deeper penetration of the PKC into the lipid bilayer and therefore the PKC-bound SAPD

fluorophore would also be positioned deeper in the bilayer. However, the motional freedom of fluorophores increases rather than decreases for a deeper position in the bilayer, which is opposite to the result found. Another possibility, that the SAPD interacts at some other non-specific site on the PKC-protein-lipid interface, can be discounted by the finding that PMA (but not 4 α -PMA) displaced SAPD completely from PKC, an effect that would not occur for non-specific interactions. Lastly, increased cholesterol concentrations, PtdEtn concentrations and unsaturation in PtdCho all increase the freedom of SAPD motion in the bilayer, producing a shorter correlation time for lipid-associated SAPD, which is opposite to the increase in the long correlation time seen for PKC-associated SAPD. We therefore conclude that the long correlation time of SAPD is associated with the conformation of the C1-domain binding pocket's changing as the enzyme becomes active; it is not sensing the bulk lipid environment. Although structural studies have been performed on isolated C1 domains [7–10,13,44,45], little other information is available on how much the C1 domain contributes to the activating conformational change. Interestingly, one study shows that a number of amino acid residues exhibit NMR line broadening on interaction of the C1B domain of PKC γ with lipids [13], supporting the idea that these domains are conformationally responsive to interactions with the membrane.

Previous studies indicate that PKC contains both high-affinity and low-affinity phorbol-ester-binding sites [29,30]; the question of the contribution of the low-affinity binding of SAPD therefore has to be considered. Insight into this can be obtained from the effect of bryostatin, which has previously been shown to compete for high-affinity binding of phorbol ester but not its low-affinity binding [29]. Accordingly, with bryostatin the three-correlation time analysis failed (two of the correlation times being identical): the analysis returned a fast (buffer-SAPD) and a slower value of 13 ns, as shown in Table 3. This value probably represents an intermediate value for SAPD bound to the low-affinity phorbol-ester-binding site and SAPD in lipids, the values being too close for the analysis to resolve. In contrast, DAG blocks low-affinity binding of phorbol ester and enhances its high-affinity binding [29]. Also consistent with this was the observation that the long rotational correlation time of SAPD was considerably increased by DAG. Increased PtdEtn concentration, cholesterol concentration or unsaturation in PtdCho, or the addition of DAG, also substantially increased activity above that induced by phorbol ester alone; again, there was a further increase in the long rotational correlation time of PKC-bound SAPD. Unlike DAG, however, the PtdEtn, unsaturation and cholesterol effects are likely to be mediated by modulation of the lipid bilayer surface energetics, which in turn modulates the PKC conformation in keeping with the results from other studies from this and other laboratories [37,38,47]. This is supported by the finding that increased physical curvature of the lipid vesicles also increases the value of the long rotational correlation time along with enzyme activity, as shown in Table 4.

Examination of the effects of increasing the concentration of Ca²⁺ provide important information on the mechanism of activation of membrane-associated PKC. Without Ca²⁺, for PKC with membrane lipids, the fraction of the long correlation time corresponding to PKC-bound SAPD is small and the corresponding activity is low, whereas at high (maximal) Ca²⁺ concentration the fraction is larger and the activity maximal. Under this condition the PKC is fully membrane-associated, as revealed by the NBD-PtdSer steady-state fluorescence anisotropy data. The finding of low activity and the corresponding appearance of the long correlation time without Ca²⁺ is expected (owing to the SAPD itself) because phorbol ester alone can

induce association with membrane and low activity [30,48]. Importantly, the value of the correlation time did not change for increased levels of Ca²⁺, whereas the activity and the fraction both showed a similar gradual increase. This is consistent with other studies showing that significantly lower concentrations of Ca²⁺ are required for half-maximal membrane binding compared with that required to activate the enzyme half-maximally [30,49]. Our interpretation is therefore that SAPD in the binding pocket of the C1 domain of PKC displays either a fast (approx. 8–10 ns) or slow (40–50 ns) rotational correlation time, corresponding to 'inactive' and 'active' conformers respectively. Thus the 'activation' event for a single PKC molecule would be a conformational change from inactive to active without a measurable contribution from intermediate states, at least with respect to the C1 activator-binding-domain region. If there were stable discrete intermediate conformations, both the value and the fraction of the rotational correlation time would also have increased gradually with increasing Ca²⁺. The results are therefore consistent with a model in which, when SAPD displays a long rotational correlation time for an individual PKC molecule, this corresponds to an 'active conformer' of PKC; increasing the Ca²⁺ concentration increases the probability that PKC will adopt an active conformation, or the proportion of active PKC conformers increases (corresponding to the increased fraction for the long correlation time).

In conclusion, we describe a model for the analysis of the time-resolved anisotropy decay of the fluorescent phorbol ester SAPD that shows that the appearance of active PKC conformers parallels the appearance of a restricted motional environment for SAPD in the C1 domain. Modifications in membrane lipids that lead to a higher PKC activity are associated with a decreased motional freedom of the phorbol ester in its PKC-binding site.

This work was supported by grants from the U.S. Public Health Service (AA08022, AA07215, AA07186 and AA074650).

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Received 28 June 1999/23 August 1999; accepted 15 September 1999