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Reorientational properties of fluorescent analogues of the protein kinase C cofactors diacylglycerol and phorbol ester

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Abstract

The reorientational properties of the fluorescently labelled protein kinase C (PKC) cofactors diacylglycerol (DG) and phorbol ester (PMA) in vesicles and mixed micelles have been investigated using time-resolved polarised fluorescence. The *sn*-2 acyl chain of DG was replaced by diphenylhexatriene- (DPH) propionic acid, while a dansyl labelled analogue of phorbol ester was used. The extent of ordering of DPH-DG in vesicles turned out to be slightly different from that of the control choline lipid DPH-PC. Addition of PKC to vesicles containing 30 mole% brain PS considerably slowed down the DPH-DG anisotropy decay. This was not observed when DPH-DG was replaced by DPH-PC. Analysis of the fluorescence anisotropy decays of these DPH-lipids in micelles polyoxyethylene-9-laurylether mixed with 10 mole% of the essential phosphatidylserine allowed estimation of their lateral diffusion, orientation distribution and reorientational dynamics within the micelles. Addition of PKC resulted in a significantly slower decay of the fluorescence anisotropy of both DPH-DG and DPH-PC even in the absence of calcium, indicating a calcium independent complexation of PKC with the PS containing micelles. Addition of calcium resulted in a further reduction of the decay of anisotropy of DPH-DG but not of DPH-PC indicating that the Ca^{2+} dependent immobilisation is cofactor-specific. Similar specific interactions with PKC resulted in a slower decay of dansylated PMA when calcium and PS were present.

Keywords: Protein kinase C; Vesicles; Micelles; Fluorimetry; Diacylglycerol; Phorbol; Reorientation

1. Introduction

The compounds diacylglycerol (DG) and phorbol esters have large regulatory effects on several cellular processes. The mechanism by which these molecules operate has been the focus of intensive study and has resulted in an increasingly detailed picture of cellular signalling. In many cases both compounds induce similar biological responses [1] which can be attributed to either activation or down regulation of protein kinase C (PKC) at the membrane level [2–6]. In addition, DG is involved in the activation of other regulatory enzymes, several phospholipases [7,8] and transmembrane proteins [9] and multiple target proteins have been found for phorbol esters [10]. Among the three PKC subfamilies, cPKC

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and nPKC respond to DG and phorbol esters while αPKC does not. Phorbol esters and DG bind similarly with one to one stoichiometry to PKC [5,11–15] and compete for binding to the kinase [11,12], although the existence of two phorbol ester binding domains has also been suggested [16]. Interaction with anionic lipids and calcium (for cPKC) is essential for the activation of PKC by phorbol esters or DG [17,18]. The precise working mechanism of calcium and its requirement for the lipid interaction of cPKC, however, remains to be elucidated [8]. Generally, it is assumed that the substrate binding pocket of inactive PKC is blocked by a so-called pseudosubstrate. Conformation changes after activation with anionic lipids, DG and calcium lead to the dissociation of the pseudosubstrate from the catalytic domain and consequently enables substrates to bind to the enzyme [19].

Biochemical studies suggest that activation of PKC by DG requires a specific stereochemical configuration of DG which indicates that the cofactors interact with specific structural elements of PKC. In addition, it was observed that only DG molecules with unsaturated acyl chains activate PKC effectively. It is unclear whether structural properties of these unsaturated chains or specific perturbations of the lipid bilayer structure induced by the unsaturated chain of DG result in the maximal stimulation of PKC (see also [8]). In some cases membrane perturbation alone can induce partial PKC activation as observed by Lester et al. [20]. It has even been suggested that the activation of PKC by DG is mainly a consequence of DG-induced changes in the physical properties of the membrane [21]. Many research groups observed that DG separates lipid headgroups in the membrane at low concentrations, whereas high concentrations of DG induce the formation of non-bilayer phases [22–25]. The relevance of non-bilayer structures in the regulation of PKC is questionable, bearing in mind that the amount of DG is low in natural membranes. For example, the DG in a rat kidney cell can rise from 0.6 mole% (normal) to 1.4 mole% (transformed) [26]. Although these studies provide valuable information about the potency of DG to modulate the membrane properties most of these studies were performed with unnatural high concentrations of DG or lipid compositions that already form quasi-stable bilayers by themselves.

In this study time-resolved fluorescence anisotropy was applied to compare the motional freedom and dynamics of diphenylhexatriene (DPH)-labelled DG and dansyl-labelled phorbol ester with those of DPH-labelled phosphatidylcholine (PC). Both sensitivity and resolution in the nanosecond time domain makes time-resolved fluorescence anisotropy an exclusive technique for detailed registration of motional dynamics and freedom of labelled lipid cofactors. The fact that the direct environment of DG is probed by its labelled acyl chain and is quantified in terms of chain dynamics and local order, adds to the previous DG studies where merely average bulk properties of the membrane were observed. In addition, the effect of PKC on the motional freedom and dynamics of probe lipids was determined from their fluorescence anisotropy decays in mixed micelles and vesicles. Vesicles are relatively large lipid systems which mimic physico-chemical properties of the biological bilayer more closely than micelles. Typically, micelles have a diameter in the nanometer range. Complexation with proteins will affect the overall rotation of the micelles and, consequently, the fluorescence anisotropy decay of lipid probes within these lipid systems. Therefore, fluorescence anisotropy forms a suitable tool to monitor association of PKC to the micellar surface. More specific interactions between the protein and cofactor will not only influence the micellar overall motion, but also the cofactor motions within the micelle. Consequently, specific interactions can be probed by measuring the fluorescence anisotropy decay of the cofactor probes in the micelle.

2. Experimental procedures

2.1. Materials

Bovine brain L-α-phosphatidylserine (PS), dioleoyl-PC (DOPC), diacylglycerol (DG), thesit (polyoxyethylene-9-lauryl ether), ethylene glycol bis(β-aminoethyl, ether)-N,N,N′,N′-tetraacetic acid (EGTA), phospholipase C from *Bacillus cereus*, phenylmethanesulfonyl fluoride, sephacryl S200, polylysine-agarose were supplied by Sigma Chemical Co (St. Louis, MO, USA). Phenyl-sepharose CL-4B and DEAE-sepharose FF S200 were from

Pharmacia (Uppsala, Sweden). All other phospholipids used were synthesised as described in the methods section. Dansyl phorbol ester and *sn*-2-(diphenylhexatriene)-PC were obtained from Molecular Probes (Eugene, OR, USA). Unless otherwise noted, experiments were performed at 277 K.

2.2. Purification of PKC

Protein kinase C was purified from the cytosolic extract of homogenised Wistar rat brains similar to the procedure described by Huang et al. [27] and consecutively by DEAE, phenyl-sepharose, sephacryl S200 and polylysine agarose chromatography. The final preparation (with isozyme composition as described elsewhere [27,28]) was essentially pure as demonstrated by silver staining of a polyacrylamide gel, and was stored at −80°C in buffer (20 mM Tris pH 7.9, 0.5 mM EGTA, 0.5 mM EDTA, 1 mM 2-mercaptoethanol) with 25% glycerol (Merck, Darmstadt, Germany; fluorescence microscopy grade).

2.3. Synthesis of DPH-labelled diacylglycerol

sn-2-(Diphenylhexatriene)-DG was synthesised by a phospholipase C catalysed hydrolysis of the diglyceride-phosphate linkage in DPH-PC as described in [29]. An incubation time of 15 min was sufficient for 100% conversion to DPH-DG. Therefore, further purification was not needed and acyl chain isomerisation of DPH-DG could be omitted. All experiments were performed with freshly synthesised DPH-DG.

2.4. Micelle preparation

Mixed lipid micelles were prepared by drying the required amounts of lipids under a stream of nitrogen in a glass tube followed by solubilisation in buffer (1 mM thesit, 20 mM Tris/HCl 120 mM NaCl and 20 μM EGTA (pH 7.4)) by vortexing and brief bath sonication. In the binding studies the thesit concentration was 100 μM and the PS concentration was 10 μM (10 mole%). The DPH-lipid concentration was 0.1 μM. The phospholipid content was determined by phosphate analysis [30]. The DPH-lipid concentration was estimated by measuring the light absorption at 355 nm in chloroform ($\epsilon = 60100\ M^{-1}\ cm^{-1}$ [31]).

2.5. Vesicle preparation

Small unilamellar vesicles were prepared by injecting 2 μl of lipid dissolved in ethanol/DMSO (dimethylsulfoxyde) through a Hamilton syringe into 200 μl of a magnetically stirred buffer solution (20 mM Tris/HCl 120 mM NaCl and 20 μM EGTA (pH 7.4)) [32]. The ratio of labelled lipid to unlabelled lipid was 1:150 on a molar basis. When the interaction of the DPH-lipids with PKC was measured, the lipid solution in ethanol was injected into buffer solution containing the protein, EGTA or calcium. With this procedure, DPH-lipid molecules located at the inner and outer membrane leaflet of the vesicles are accessible for PKC.

2.6. Time-resolved fluorescence and fluorescence anisotropy measurements

The polarised fluorescence decays were measured by use of a time-correlated single photon counting set-up as described elsewhere [33]. The excitation wavelength was 340 nm. The fluorescence emission of the probes was selected using a KV 399 cut-off filter (Schott, Mainz, Germany) and an interference filter at 441.7 nm (Schott, bandwidth 10.9 nm full width half maximum (FWHM)) for the DPH lipids and a KV 500 cut-off filter (Schott) for dansyl labelled phorbol ester. After each sample the background of samples in the absence of DPH-lipids was measured for correction of the sample polarised decays. 2,5-Diphenyloxazole (Eastman Kodak Co.) dissolved in ethanol served as reference compound ($\tau = 1.6$ ns [34]) to yield the dynamical instrumental response function of the set-up [35]. The samples were thermostated at 277 K.

2.7. Data analysis

By measuring the time-dependent parallel and perpendicular polarised emission components $I(t)_{\parallel}$ and $I(t)_{\perp}$ relative to the polarisation direction of the exciting beam one can recover the fluorescence anisotropy $r(t)$:

$$I(t)_{\parallel} = P(t) \times \left(\frac{2}{3} r(t) f(t) + \frac{1}{3} f(t) \right) \quad (1)$$

$$I(t)_{\perp} = P(t) \times \left(\frac{-1}{3} r(t) f(t) + \frac{1}{3} f(t) \right). \quad (2)$$

The multiplication sign indicates the convolution of the decay function with the instrument impulse response profile $P(t)$. $f(t)$ is the intrinsic fluorescence decay function and $r(t)$ the anisotropy decay function which is described by the following time correlation function [36]:

$$r(t) = \frac{2}{5}\langle P_2[\,\mu_a(O)\,\mu_e(t)]\rangle \qquad (3)$$

where the factor 2/5 accounts for the maximum theoretical anisotropy as a result of photoselection, $P_2[\,\mu_a(0)\mu_e(t)]$ is the second-order Legendre polynomial, $\mu_a(0)$ is the direction of the absorption transition moment in the molecular frame at zero time and $\mu_e(t)$ is the direction of the emission transition moment at time t. The brackets $\langle . \rangle$ denote an ensemble average. Each process that results in a more uniform distribution of μ_e will give a decrease of the anisotropy of the emitted light. Therefore the molecular order and rotational dynamics of probe molecules in lipid systems can be determined from the relaxation of the anisotropy [37,38]. When lipid probes are dispersed in micelles, three independent motional processes may contribute to the observed anisotropy decay: (1) internal rotation of the lipids within the micelles, (2) lateral diffusion of the lipid probes within the micelle and (3) overall rotation of the micelle. In larger lipid systems like vesicles only rotational motion of the lipid probes within the bilayers contribute to the anisotropy decay. The rotational diffusion model (r_{g3}) describes the depolarisation induced by rotational motion of probes like DPH in terms of a perpendicular diffusion coefficient $D_\perp$ and the two order parameters $\langle P_2 \rangle$ and $\langle P_4 \rangle$ [37,38]. The parameters $\langle P_2 \rangle$ and $\langle P_4 \rangle$ are related via an equilibrium orientation distribution function $f(\theta)$ of the DPH labels with respect to the membrane normal:

$$\langle P_2 \rangle = \frac{1}{N}\int_0^\pi f(\theta)\,P_2(\cos\theta)\sin\theta\,\mathrm{d}\theta \qquad (4)$$

$$\langle P_4 \rangle = \frac{1}{N}\int_0^\pi f(\theta)\,P_4(\cos\theta)\sin\theta\,\mathrm{d}\theta \qquad (5)$$

$$N = \int_0^\pi f(\theta)\sin\theta\,\mathrm{d}\theta. \qquad (6)$$

When no assumptions are made about the orientation distribution, $\langle P_2 \rangle$ and $\langle P_4 \rangle$ are determined independently. By applying the maximum entropy formalism, the most unbiased orientation distribution function can be constructed from optimised $\langle P_2 \rangle$ and $\langle P_4 \rangle$ values [39,40]. This approach, however, often yields bimodal orientation distribution functions for DPH, even when the labels are covalently attached to lipids [33]. Such an orientational heterogeneity is not consistent with the known physicochemical properties of DPH-lipids and membrane system. Apparently, in this approach a lowering of $\langle P_2 \rangle$ is interpreted to arise from an increase in the perpendicular oriented population of probes. The compound motion model, recently adapted for fluorescence anisotropy decay analysis of DPH-like probes, seems to be a more appropriate approach for analysis [41], but contains 5 adjustable parameters which restricts its application to macroscopically oriented lipid systems. Therefore, we analysed the anisotropy decay originating from rotation of DPH-lipids in micelles and vesicles according to the r_{g3} model with the intuitive assumption that the orientation of the DPH-labels is Gaussian distributed parallel with respect to the direction of the phospholipid acyl chains with distribution width θ_g (see also [42]):

$$f(\theta) = \mathrm{e}^{-(\theta/\theta_g)^2} + \mathrm{e}^{-((\pi-\theta)/\theta_g)^2}. \qquad (7)$$

Upon binding of PKC to vesicles, two populations of DPH-lipids might arise with different orientation distributions. One fraction (1-β) of free DPH-DG molecules will remain in the bulk membrane with an orientation distribution width (θ_{gf}) similar to the situation without addition of protein. A second fraction β of the DPH-DG molecules will interact with PKC with unknown orientation distribution (θ_{gb}).

$$f(\theta) = \beta\left[\mathrm{e}^{-(\theta/\theta_{gb})^2} + \mathrm{e}^{-((\pi-\theta)/\theta_{gb})^2}\right] + (1-\beta)\left[\mathrm{e}^{-(\theta/\theta_{gf})^2} + \mathrm{e}^{-((\pi-\theta)/\theta_{gf})^2}\right]. \qquad (8)$$

Global analysis of multiple DPH anisotropy decays measured at various concentrations of PKC with the bimodal distribution of DPH-probes (Eq. 8) will then recover the fraction of DPH-DG molecules interacting with PKC (β), and the angular distribution of the bound cofactor molecules (θ_{gb}).

In micelles, the anisotropy decay curves of the DPH-lipids in micelles were analysed by a product

of three correlation functions corresponding to the overall motion of the micelle with rotational correlation time ϕ_r, the probe lateral motion expressed by translational correlation time ϕ_T and the probe internal motion described by $D_\perp$ and θ_g in the r_{g3} model:

$$r(t) = e^{-t/\phi_r} e^{-t/\phi_T} C(r_0, D_\perp, \theta_g, t) \quad (9)$$

The correlation time ϕ_T is related to the lipid lateral diffusion D_L by:

$$\phi_T = \frac{r_m^2}{4D_L} \quad (10)$$

where r_m corresponds to the radius of the micelle. In these analyses ϕ_r was determined independently from translational diffusion of micelles (coefficient D_m in $m^2\ s^{-1}$) measured with fluorescence correlation spectroscopy [43]. In the present calculations the rotation of micelles are assumed to be isotropic. While this assumption may not be realistic for micelles complexed with PKC, it is unlikely to introduce a serious error. The correlation time ϕ_r is then related to D_m by:

$$\phi_r \approx \frac{6.25 \cdot 10^{-4} k^2 n_2 T^2}{n_1^3 D_m^3} \quad (11)$$

where k, T, n_1 and n_2 correspond respectively to the Boltzmann constant, temperature and viscosity of the sample measured with correlation spectroscopy and the sample measured with time correlated single photon counting ($n_1 = 17.4$ and $n_2 = 11.1$ mPa s). The parameters ϕ_T, $D_\perp$ and θ_g were determined for each experiment independently while the initial anisotropy, r_0, was adjusted globally from multiple experiments. When the DPH-lipids were dispersed in vesicles, ϕ_r and ϕ_T were fixed to large values in the ms range.

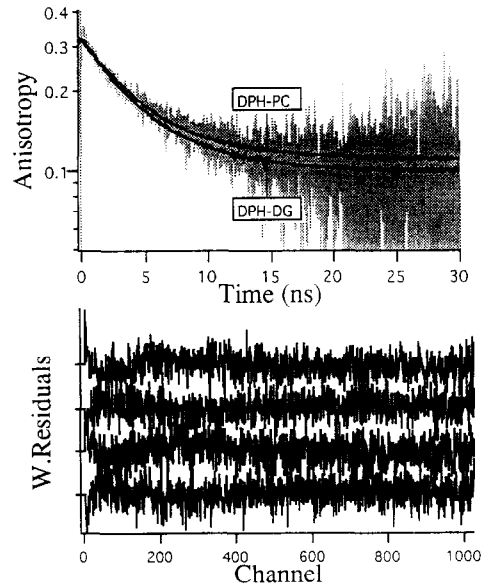

Fig. 1. Experimental and fitted anisotropy decays of DPH-DG and DPH-PC in DOPC vesicles. The smooth curve corresponds to the optimal fit with the r_{g3} model assuming a Gaussian orientation distribution. In the lower panel the weighted residuals of the fit to the perpendicular and parallel decay curves of DPH-PC (upper two curves) and DPH-DG (lower two curves) are presented.

3. Results

3.1. Orientation distribution of DPH analogues of DG and PC in DOPC vesicles

In Fig. 1 the experimental anisotropy decays of DPH-DG and DPH-PC in DOPC vesicles are pre-

Table 1
Optimised θ_g and $D_\perp$ values from analysis of the data sets of DPH lipid probes in DOPC vesicles using the r_{g3} model

Probe	System	θ_g	$\langle P_2 \rangle$	$\langle P_4 \rangle$	$D_\perp$ $(\mu s)^{-1}$	χ^2
DPH-DG	DOPC	0.63 (0.62–0.65)	0.56	0.15	28 (26–32)	1.21
DPH-PC	DOPC	0.59 (0.57–0.61)	0.60	0.18	27 (25–32)	1.23

The initial anisotropy was linked over all experiments and amounts to 0.323. The numbers between parentheses correspond to the errors as determined from a rigorous error analysis at a 67% confidence level. The $\langle P_2 \rangle$ and $\langle P_4 \rangle$ values were calculated from θ_g using Eqs. 4 and 5.

sented. The smooth curves through the data points represent the optimised fits according to the $r_g 3$ model with the assumption that the orientation distribution of the DPH-lipids is Gaussian shaped. It can be seen that the motional constraints of DPH-DG and DPH-PC in these fluid state membranes differ slightly. In the lower panel of Fig. 1 the weighted residuals of the fit to the parallel and perpendicular fluorescence decay curves of both DPH lipids are given. As can be concluded from the random scattering of the residuals the model adequately fits the decay curves of both DPH-lipids at longer times. At shorter times, however, the model is not able to perfectly match the experimental data, which results in underestimation of the initial anisotropy r_0. Therefore this approach is compromising the advantage of a minimal number of adjustable parameters ($D_\perp$, θ_g) and an intuitively correct probe orientation with a sub-optimal fit to the experimental data.

No significant differences in the fluorescence decay characteristics were observed for the different DPH-lipids (data not shown), which is not surprising bearing in mind that the DPH moiety is linked identically to both lipids. In Table 1 the recovered θ_g and $D_\perp$ values, and corresponding $\langle P_2 \rangle$ and $\langle P_4 \rangle$ values (calculated from θ_g according to Eqs. 4 and 5) are presented. Since both DPH-PC and DPH-DG closely mimic their natural lipids, the probe order parameters will reflect the overall molecular ordering experienced by their natural analogues in the membrane. From the experimental plots and the recovered parameters listed in Table 1 it can be concluded that when DPH is attached to DG its fluorescence anisotropy decays to lower values than when attached to PC. The differences are, however, small. This observation indicates a lower degree of ordering of the neighbouring lipid chains experienced by DPH-DG. The lack of the phosphocholine headgroup in the DG host lipid apparently induces a packing failure of a close fit to the neighbouring lipid chains which results in slightly more angular freedom.

3.2. Interaction between PKC and DPH-DG in a vesicular lipid system

In order to investigate the effect of interaction with PKC on the rotational properties of DG and PC in DOPC/PS vesicles, the polarised decays of both

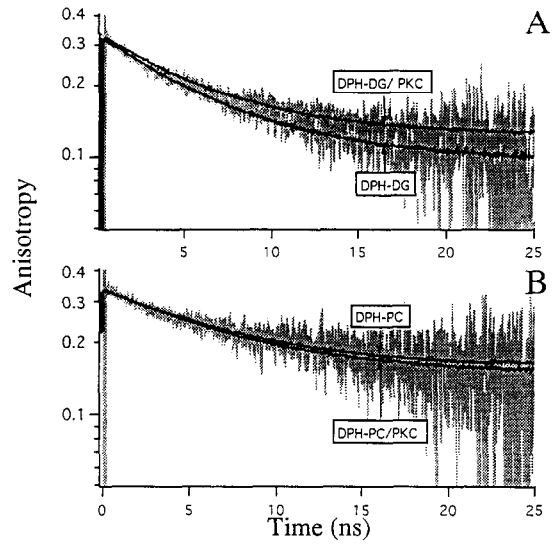

Fig. 2. Experimental anisotropy decays of DPH-DG (A) and DPH-PC (B) in vesicles of DOPC/PS (80:20) in the presence and absence of PKC (0.4 μM). In the blank experiments without PKC, the dialysis buffer of PKC was added to the vesicles. The smooth curve corresponds to the optimal fit with the r_{g3} model assuming a Gaussian orientation distribution.

DPH-lipids were measured in the presence of various concentrations of PKC. To facilitate PKC binding to DPH-lipids located at the inner and outer leaflets of the membrane, the vesicles were prepared by injecting small aliquots of the mixed lipids in ethanol into buffer solutions with or without PKC. The experimental fluorescence anisotropy decays of DPH-DG and DPH-PC in the absence and presence of 0.4 μM PKC are given in Fig. 2A and 2B, respectively.

The fluorescence anisotropy of DPH-DG decays to higher residual values when PKC is present than when it is absent, indicating additional motional restriction of the labelled cofactor when it interacts with PKC. On the contrary, the fluorescence anisotropy decay of DPH-PC is not affected by the presence of PKC. Apparently, the effects of PKC are specific for DPH-DG and cannot be explained by a more general effect of interacting PKC molecules on the vesicle properties.

The difference in the anisotropy decays in the presence and absence of PKC indicates a distinct difference between the motional properties in the protein boundary and lipid bilayer regions. Since the only difference between the labelled DG and PC is

Table 2
Optimised θ_g and $D_\perp$ values from analysis of the data sets of DPH lipid probes in DOPC/PS (8:2) vesicles using the r_{g3} model

Probe	System	θ_g (rad)	$\langle P_2 \rangle$	$\langle P_4 \rangle$	$D_\perp$ (μs)$^{-1}$	χ^2
DPH-DG	PS/PC/Ca^{2+}	0.65 (0.64–0.68)	0.53	0.12	27 (25–29)	1.19
	PS/PC/Ca^{2+}/PKC	0.54 (0.52–0.57)	0.65	0.24	20 (19–22)	1.26
DPH-PC	PS/PC/Ca^{2+}	0.60 (0.59–0.61)	0.59	0.17	25 (24–27)	1.19
	PS/PC/Ca^{2+}/PKC	0.60 (0.59–0.61)	0.59	0.17	27 (25–29)	1.17

The initial anisotropy was linked over multiple experiments and amounts to 0.323. In the experiments 0.5 mM calcium and 0.4 μM PKC were used. The total lipid concentration was 15 μM. See for further details the legend of Table 1.

the lack of the phosphocholine headgroup (note that DPH-DG was synthesised from DPH-PC) this result indicates that specific chemical features of the DPH-DG headgroup provides the interaction with PKC. Alternatively, specific physico-chemical features of the DPH-DG lipid environment may be recognised by PKC. Analysis of the anisotropy decays with the r_{g3} model with the assumption of a unimodal probe orientation (Eq. 7) yielded optimised values for θ_g and $D_\perp$ (Table 2).

It is obvious from these θ_g values that in presence of PKC, the DPH moiety attached to DG is oriented with smaller angles to the membrane normal than in absence of PKC. In addition, the rotational dynamics of DPH-DG is slightly reduced by PKC. It is important to note that the optimised θ_g value obtained in presence of PKC reflects the average motional freedom of DPH-DG molecules which are interacting with PKC and of those which are free in the bulk membrane. To derive an indication of the orientation distribution of the DPH-DG probes bound to PKC separate from those in the bulk lipid, the anisotropy decay curves of DPH-DG at various concentrations of PKC were analysed globally with a weighted sum of two Gaussian distributions (Eq. 8). In this analysis, the Gaussian width of non-interacting DPH-DG molecules (θ_{gf}) was fixed to 0.65 as obtained without PKC (Table 2). In the analysis of ten fluorescence anisotropy decays obtained at different PKC concentration, the angular distribution of the bound cofactor molecules (θ_{gb}) and $D_\perp$ were adjusted globally, while the fraction of DPH-DG molecules interacting with PKC (β) was recovered individually for each experiment. The analysis yielded an optimised value of 0.41 for θ_{gb}. As can be expected from a multiparameter fit, the uncertainty in θ_{gb} is relatively large (0.26–0.54). Therefore, this analysis should be considered as an approximate one. The weighted orientation distributions of the probes with respect to the membrane normal (which represent the probability of finding the probe at a certain angle) were constructed from the optimised θ_{gf} and θ_{gb} values (Fig. 3A). The fractional contribution β of DPH-DG with Gaussian width θ_{gb} as a function of

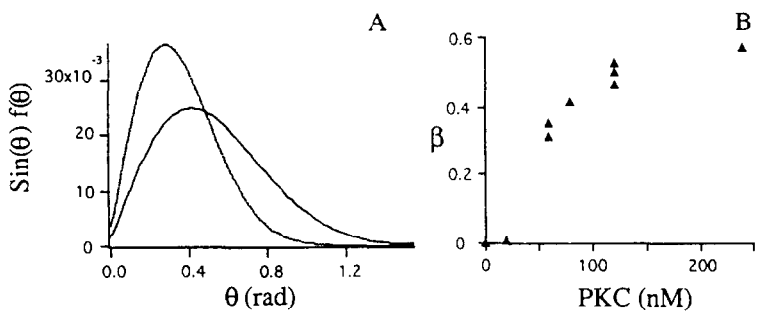

Fig. 3. (A) Orientation distributions of probe molecules with Gaussian width of 0.65 (—) and 0.41 (- - -). These values correspond to the optimised values of free and PKC-bound DPH-DG, respectively. (B) The fractional contribution (β) of bound DPH-DG with Gaussian width θ_{gb} as a function of PKC concentration. The orientation distribution of DPH-DG was assumed to be composed of two Gaussian distributions of free and bound DPH-DG as described in the Experimental procedures. In this analysis the motional freedom of the free DPH-DG lipids θ_{gf} was fixed to the value obtained in the absence of PKC. The total lipid concentration was 15 μM.

the PKC concentration is plotted in Fig. 3B. It can thus be concluded that the DG acyl chains are restricted in their motion upon specific interaction with PKC.

3.3. Interaction between PKC and DPH-DG in a mixed micellar lipid system

In order to investigate the role of PS and calcium in the PKC-DG interaction, the anisotropy decays of DPH-DG and DPH-PC dispersed in micelles were determined at various binding conditions. Since PKC and thesit micelles have approximately similar sizes, the interaction of PKC to micellar lipids is expected to influence multiple independent motions; the isotropic rotation (ϕ_r) of the relatively small micelles and the translational diffusion (ϕ_T) and rotational motion of the probes (θ_g, $D_\perp$) around the radial vector of the micelle. Evaluation of each individual motional component at various PKC-lipid binding conditions will yield information which is difficult to obtain with vesicles. In Fig. 4A the fluorescence anisotropy decays of DPH-DG in thesit micelles containing 10 mole% PS are presented. The fastest decay corresponds to DPH-DG in the absence of PKC. From visual comparison of this decay with the one obtained in vesicles (Fig. 1) one can conclude that additional slow rotational motions are registered by DPH-lipids in micelles which result in a continuous decay of the anisotropy, even at longer times. These slow depolarisation processes originate from micellar rotation and lateral motion of lipids within the micelle. The rotational correlation time of micelles in absence PKC was calculated from translational diffusion coefficients obtained from fluorescence correlation spectroscopy [43] and amounts to

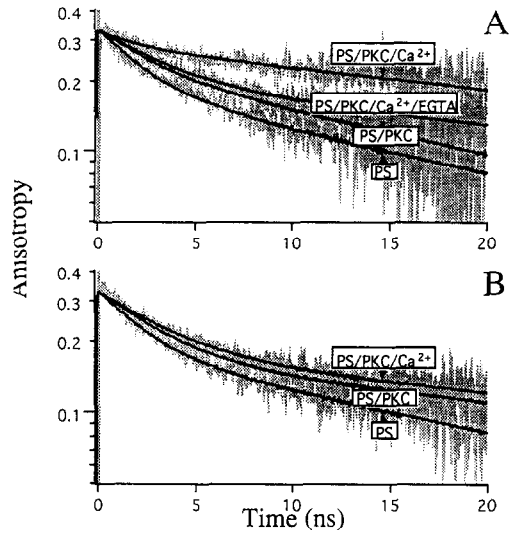

Fig. 4. Experimental anisotropy decays of DPH-DG (A) and DPH-PC (B) in thesit micelles containing 10 mole% PS at various binding conditions (see text).

37 ns for free micelles (Table 3). Under approximation of spherical micelles this correlation time corresponds to a Stokes radius of 3.1 nm. No significant differences are observed between the experimental decays of DPH-DG and DPH-PC in absence of PKC (Fig. 4B). This is not surprising, since the micellar rotation and lipid translational motion are mainly determined by the properties of the micelle itself and thus insensitive to DPH-lipid species. Global analysis of the anisotropy decay curves of DPH-DG and DPH-PC, in which r_0 was linked and ϕ_T and θ_g were determined from the individual decays of the DPH-lipids, yielded optimised values of 84 and 88 ns for ϕ_T and ca. 0.46 radians for θ_g (Table 3). The

Table 3
Optimised ϕ_T, θ_g and $D_\perp$ values from analysis of the data sets of DPH lipid probes in thesit micelles

Probe	System	ϕ_r (ns)	ϕ_T (ns)	θ_g (rad)	$\langle P_2 \rangle$	$\langle P_4 \rangle$	$D_\perp$ (μs)$^{-1}$	χ^2
DPH-DG	PS	37	84 (58–340)	0.46 (0.43–0.52)	0.73	0.35	29 (26–33)	1.13
	PS/PKC	63	112 (85–400)	0.43 (0.41–0.47)	0.76	0.40	28 (26–32)	1.21
	PS/PKC/Ca^{2+}	98	103 (80–400)	0.22 (0.20–0.31)	0.93	0.78	12 (10–18)	1.19
DPH-PC	PS	37	88 (60–320)	0.46 (0.44–0.52)	0.73	0.35	29 (27–32)	1.14
	PS/PKC	63	120 (73–∞)	0.46 (0.43–0.51)	0.73	0.35	24 (22–29)	1.18
	PS/PKC/Ca^{2+}	98	126 (75–∞)	0.43 (0.41–0.48)	0.75	0.38	21 (19–24)	1.21

The initial anisotropy was linked over multiple experiments and amounts to 0.325. The ϕ_r values were calculated from the translational diffusion coefficients of micelles independently measured by fluorescence correlation spectroscopy [43]. See for further details the legend of Table 1.

errors of ϕ_T and θ_g at the 67% confidence level, determined with an error analysis indicate that the differences between the θ_g values of both DPH-lipids are within the error of the experiment. The translational diffusion coefficient is related to ϕ_T (Eq. 10) and amounts to $6.5 \cdot 10^{-11}$ m^2 s^{-1} which is larger than values obtained for lipids in bilayer membrane with pyrene excimer experiments (ca. 10^{-11} m^2 s^{-1} [44]).

Addition of PKC leads to slower decay of the anisotropy of both DPH-DG (Fig. 4A) and DPH-PC (Fig. 4B) even in the absence of calcium (50 μM EGTA). This observation indicates a calcium independent interaction of PKC with the micelles. In these experiments, with a thesit concentration of 100 μM, micelles are composed of approximately 300 detergent molecules [45]. Thus on the average one out of three micelles contains a DPH-lipid molecule, and at a PKC concentration of 0.4 μM, the protein is present in approximately 1.5-fold excess over the micelles. No effect of PKC on the DPH-DG anisotropy decay is observed when 10 mole% PS is replaced by 10 mole% PC in thesit micelles (data not shown) confirming that PS is a prerequisite for the interaction of PKC with a membrane surface [46,47]. Conversely, mixed micelles containing 5 mol% PS are fully capable to bind PKC in the presence of calcium. Probably calcium is needed for the interaction of PKC with PS. Binding of PKC to PS molecules precedes the interaction with lipid cofactors which then leads to activation of PKC.

From correlation spectroscopy, we obtained a value of $\phi_r = 63$ ns for the micellar rotation under these experimental conditions [43]. Analysis of the experimental fluorescence anisotropy decays (Eq. 9) yielded optimised values for ϕ_T and θ_g. As is shown in Table 3, in absence of calcium, PKC mainly influences the overall rotation of the micelle and the translational diffusion of the lipids within the micelle. No significant differences were obtained between DPH-PC and DPH-DG indicating that in this stage of PKC-association to PS, hardly any specificity is obtained for DPH-DG. Addition of calcium leads to a further slowing down of the anisotropy of both DPH-PC and DPH-DG. In this case, however, large differences are obtained between the anisotropy decays of DPH-DG and DPH-PC, indicating that calcium enhances binding of PKC to PS containing membranes. No significant effects of calcium on the anisotropy decays of both lipids were observed in absence of PKC.

In presence of PKC and calcium a correlation time of 98 ns was derived for the rotation of the micelles from its translational diffusion [43]. Analysis of the anisotropy decays (Eq. 9) yielded optimal values for ϕ_T, θ_g and $D_\perp$ (Table 3). Apparently, in the presence of calcium PKC interacts with higher affinity to the PS containing micelles which results in a further reduction of the overall rotational motion of the micelles. No significant effects were observed of calcium on the translational diffusion (note that for a probe with a fluorescence lifetime of 10 ns a correlation time longer than 100 ns seems to be already infinitely long). Next to this general complex formation between PKC and micelles, a strong reduction of the θ_g and $D_\perp$ values of DPH-DG is observed when calcium is added, indicating that the rotational freedom and dynamics of DPH-DG interacting with PKC is very limited. When DPH-PC is used instead of DPH-DG, these lipid motional parameters are hardly influenced indicating that only in the presence of calcium specificity is obtained for DPH-DG. This calcium modulation of lipid specificity can be explained by statistical and/or allosteric mechanisms.

The statistical explanation is based on similar mechanism as was proposed to explain the sigmoidal dependencies of PKC binding and activity on the PS mole fraction in membranes [19,48,49]. The probability of DG binding to PKC increases with the probability of finding PKC at the micellar surface. In absence of calcium PKC is only loosely bound to the micelles, dynamically exchanging between buffer and micellar surface. Under these binding conditions, the time that the protein is retained at the micellar surface is too short for interaction with DG. When calcium is added, the protein becomes more firmly bound to the micellar surface (as can be concluded both from fluorescence correlation spectroscopy and time-resolved fluorescence anisotropy) resulting in a higher probability of interaction with DG.

The second alternative option is that in absence of calcium PKC is already bound to the micellar surface but for unknown reasons it is not able to interact with DPH-DG. Calcium does not only change the binding characteristics of PKC to PS but also affects

the protein in such a way that direct interaction with DPH-DG is possible. In this context one might hypothesise that inactive PKC is not able to bind DG. Binding of calcium to PKC allosterically affects the protein conformation thereby facilitating binding of DPH-DG. The interaction of DG with PKC stabilises the active form of PKC. Distinguishing between these two mechanisms has to be done in further investigations.

3.4. Interaction of PKC with dansyl labelled phorbol ester in vesicles and micelles

In order to investigate the effect of PKC on the reorientational properties of phorbol esters, we performed similar experiments with dansyl labelled phorbol myristate acetate (PMA) as described above for DPH labelled lipids. In the absence of PS no effects of PKC on the anisotropy decay of dansyl labelled PMA could be detected indicating that like in the case of DG, PS is a prerequisite for the interaction of PKC with phorbol ester. In the presence of PS and calcium large effects were observed on the anisotropy decay of this labelled compound when PKC was added. The anisotropy decay curves of dansyl-PMA in micelles containing 10 mole% PS in the presence and absence of PKC are given in Fig. 5. Specific trends of PKC on the motional properties of the labelled PMA were detected, analogous to those of DPH-DG. Addition of a four-fold excess of unlabelled PMA yielded the same anisotropy decay of dansyl-PMA as the one obtained in absence of PKC indicating a reciprocal relationship of labelled and unlabelled PMA with respect to PKC binding.

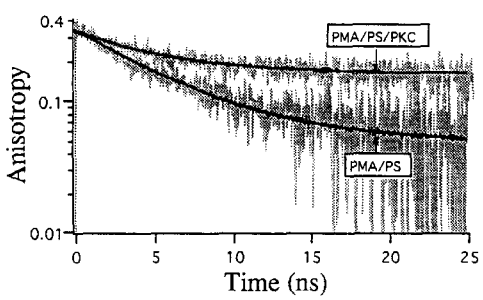

Fig. 5. Anisotropy decays of dansyl-PMA in mixed micelles in the presence and absence of PKC. The micelles were composed of thesit, 10 mole% PS. The smooth curve through the data points represents an optimised fit with a model consisting of a sum of three exponentials.

4. Discussion

Motional properties of fluorescent cofactors of protein kinase C have been characterised in vesicles and micelles by monitoring the decay rate of the fluorescence anisotropy. Applied to micelles, this technique enables registration of the binding of PKC to PS containing micelles. In addition, specific interaction of PKC can be observed from anisotropy decay patterns of labelled cofactors in vesicles and micelles. In this sense both membrane-mimetic systems reveal complementary information about the general binding to membrane and subsequent interaction with cofactors. The rotational freedom of DPH-DG in DOPC vesicles is slightly larger than that of DPH-PC while the rotational dynamics of both probes are approximately similar. The lack of the phosphocholine headgroup of DPH-DG apparently influences the organisation of its lipid neighbours in such a way that additional motional freedom is obtained. The probable failure of DPH-DG to be tightly accommodated in the lattice of surrounding lipid chains would allow more extended chain motions than for DPH-PC which is arranged in a complementary manner to DOPC lipids. Organisational defects in the membranes induced by DG may result in exposure of hydrophobic lipid elements to the aqueous environment which increases the ground state energy of a bilayer. Consequently the lipid packing irregularities will decrease the apparent activation energy change associated with incorporation of proteins like PKC [50]. The incorporation of PKC may precede or even be strictly required for direct (stereospecific) interactions with DG that further stabilise the active PKC. An alternative explanation for the small variance in motional freedom of DG and PC might be a difference in the distribution of both lipids in the outer and inner membrane leaflet.

The fact that in presence of EGTA, the rotation and translational diffusion of PS containing micelles slows down considerably upon addition of PKC shows that this protein is able to interact with PS containing membranes in the absence of calcium (see also [51–53]). Addition of calcium enhances the

interaction of PKC with PS containing membranes and results in binding of PKC to DG. Apparently, the binding of PKC to PS containing membranes is stabilised by calcium. Several other studies have provided evidence that calcium is essential for association of PKC with phospholipid membranes [3,4,54] and significant conformational rearrangements of PKC have been observed upon its interaction with calcium and upon the binding of PKC to membranes [55]. Both in micelles and vesicles the presence of PKC and calcium leads to a reduction of the motional freedom of DPH-DG, whereas its rotational dynamics is barely affected. We can therefore conclude that the motion of the DG chains at the protein surface is restricted. The fact that the fluorescence properties of DPH-DG barely change when it interacts with the protein indicates that the dielectric constant of the DPH environment is invariant upon interaction. Therefore movement of DPH-DG from the hydrophobic membrane core to a more hydrophilic environment accompanied with binding to PKC is not likely. It is more probable that DPH-DG interacts in a dynamical fashion with the protein surface within the hydrophobic membrane or even is trapped in grooves in the polypeptide.

These results imply that in resting cells, cPKC will interact with the plasma membrane and intracellular membranes but not with its cofactor DG (which is even present in low amounts in resting cells [26]). Release of the intracellular calcium levels upon a stimulus of cells will enable the kinase to interact more firm with the PS containing membranes and with DG. Whether the calcium dependence of DG binding to PKC is a direct consequence of the enhanced binding of PKC to PS membranes or a result from allosteric effects induced by calcium that result in exposure of a shielded lipid cofactor site for DG is unclear. Insertion of PKC into membranes has been observed by several research groups [52,3]. It is tempting to explain the calcium dependence of binding to PS and DG in terms of a calcium induced shift from loosely peripheral bound PKC to inserted PKC. The hydrophobic interaction forces between the inserted PKC segments and lipid acyl chains will add to the electrostatic interactions between the protein and PS headgroups and may induce structural rearrangements that enable binding of DG to PKC. On its turn, the binding of DG may stabilise the inserted active form of PKC and will thus lower the calcium requirement for the activation of PKC as was observed by Kishimoto et al. [56] and several others.

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