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Fluorescence Methods to Study Lipid–Protein Association: The Interaction of Protein Kinase C with Lipid-Loaded Mixed Micelles

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The interaction of protein kinase C with lipids was studied in a mixed micellar system. Two fluorescence spectroscopic methods are presented with a different but complementary information content. Diffusion monitored by fluorescence correlation spectroscopy provides information on the interaction of the protein with the whole lipid aggregate. Resonance energy transfer from tryptophans to pyrene-labeled lipids monitored by time-correlated single-photon counting supplies information on the interaction of the protein with specific lipid cofactors within the micelle. The results can be extended to postulate new mechanisms for the activation of protein kinase C by the signal transduction cascades in the cell. Both fluorescence spectroscopic methods can be easily applied to other protein systems which interact with lipids.

KEY WORDS: Lipid–protein association; protein kinase C; mixed micelles.

INTRODUCTION

Extracellular chemical signals as a result of ligand–receptor interactions can be transferred to the inside of the cell by protein–protein interactions or protein–lipid interactions. In the latter case the cell surface signal is transduced by enzymatic chemical modification of specific lipid molecules, which can then interact with and activate a specific protein such as a kinase. A classical example of such a signal cascade is the activation of protein kinase C (PKC) [1–6]. Ligand–receptor interaction results in the activation of phospholipase C (PLC) mediated by a g-protein through protein–protein interactions. The activated PLC hydrolyzes phosphoinositidebiphosphate (PIP_2) in inositidetriphosphate (IP_3) and diacylglycerol (DG), resulting in two molecular messages: one in the cytosol (IP_3) and one in the membrane (DG) [6–10]. The cytosolic second messenger IP_3 binds to calcium channels on the endoplasmic reticulum (ER), giving rise to up to a 10^4-fold increase in cytosolic calcium levels. The increased calcium levels induce a translocation of PKC from the cytosol to the membrane, where it interacts with negatively charged phosphatidylserine (PS) [11, 12] and is activated by DG (Fig. 1).

In order to study lipid–protein interactions a suitable model system must be chosen which will form a stable molecular aggregate with the lipids of interest. The choices within the category of macroscopic isotropic systems are vesicles or mixed micelles [11]. Vesicles have the advantage that they mimic the biological bilayer structure with its physicochemical properties. The disadvantage of these systems is that binding stoichiometries of protein and lipids are difficult to determine and the structural complexity of the system can add physical parameters to the binding model which are difficult to control (e.g., surface curvature, phase). Micelles

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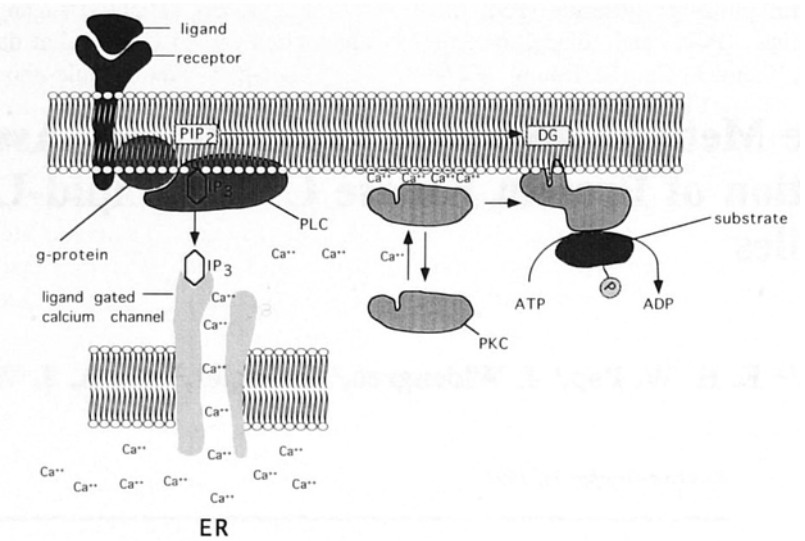

Fig. 1. Schematic drawing of the classical signal pathway to activate PKC within a cell. See text for details.

have the advantage that they form a simple experimental system in which parameters such as size and stoichiometries of interacting components are easily controlled [13, 14]. The drawback of this simplicity is that micelles do not mimic the biological bilayer structure with its physicochemical properties. We studied the interaction of PKC with its lipid cofactor DG and "upstream" lipid precursors PIP_2, PIP, PI, and PC in order to determine if any of these lipids can bind and activate PKC [14]. When interactions of a protein with lipids of different chemical structure such as described above are compared, mixed micelles are the system of choice since other than chemical factors are of a minor concern.

The most evident way of measuring association of a protein with a lipid aggregate is by a physical observable which is related to size of particles. Both rotational and translational diffusion are, on first approach, inversely proportional to the volume of the particle and can be measured by fluorescence spectroscopic techniques provided that an extrinsic fluorophore is present in the system. In this article an example is given where fluorescence correlation spectroscopy is used to measure calcium-dependent lipid–PKC interactions by monitoring translational diffusion of NBD-PE-labelled micelles.

Förster energy transfer is another method to detect protein–lipid interactions since it is a short range dipole–dipole interaction which operates when (energy) donor and acceptor are in close proximity (1–10 nm). In this case Förster energy transfer from UV-excited intrinsic tryptophans residues in the protein to acceptor probes in the lipid aggregates can be detected by steady-state fluorometry or time-resolved fluorescence spectroscopy. In a time-resolved experiment energy transfer can be directly monitored by the decrease in the average lifetime of the donors due to the additional process of nonradiative decay. The advantage of this method is that it does not suffer from artifactual inner filter effects which affect the fluorescence intensity. The interaction of PKC with vesicles has been studied by steady-state fluorescence spectroscopy, where energy transfer is monitored by the decrease in the donor (8 tryptophans in PKC) emission or the increase in sensitized acceptor (labeled lipid) emission [15–19]. In these studies an acceptor fluorescent lipid probe is introduced in the vesicles which is believed not to interact with PKC. In this way only the binding of PKC with vesicles can be detected, and not the specific interaction with lipid cofactors within the lipid aggregates. In order to study the latter interaction the lipid cofactors must be chemically modified such that they contain an intrinsic acceptor probe. For this purpose we have synthesized pyrene-labeled DG (pDG), PIP_2 ($pPIP_2$), PIP (pPIP), and PI (pPI) by attaching a fluorescent pyrenedecanoic acid to the *sn*-2 position of the lipid glycerol [14]. The fluorescent pyrene moiety is an excellent energy acceptor of singlet excited tryptophans, with an approximate critical transfer distance of 2.7 nm.

MATERIALS AND METHODS

PKC was isolated from 30 Wistar rat brains according to the procedure described by Huang *et al.* [20]. The protein was > 95% pure as judged by silver-stained SDS-PAGE, with a specific activity of 300 units/mg at

20°C [13]. Bovine brain phosphatidylserine (PS), dioleoyl-phosphatidylcholine (PC), and diacylglycerol (DG) were from Sigma Chemical Co. (St. Louis). *N*-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)-1, 2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine, triethylammonium salt (NBD-PE), was obtained from Molecular Probes. Fluorescently labeled DG (pDG), PIP2 (pPIP2), PIP (pPIP), and PI (pPI) were synthesized as described elsewhere [14]. Stock solutions of mixed micelles were prepared by evaporating chloroform from a solution of lipids under a stream of dry nitrogen. The lipid film was resolubilized in 1 m*M* Thesit (polyoxyethylene 9-lauryl ether; obtained from Sigma), 20 m*M* Tris, pH 7.5, 100 m*M* NaCl. Of this stock solution a fixed volume was added to a reaction mixture containing a variable amount of PKC such that the final concentration of the detergent was 100 μ*M* Thesit in 20 m*M* Tris, pH 7.5, 100 m*M* NaCl, 0.1 m*M* EDTA, 0.1 m*M* EGTA. The reaction mixture was allowed to equilibrate for 15 min at 20°C before a measurement.

Fluorescence correlation spectroscopy (FCS) was done with an apparatus as described by Rigler *et al.* [21]. The 457.9-nm line of an argon-ion laser was used to excite NBD-PE. The laser intensity was attenuated by a neutral density filter with an OD of 3.0, to give a maximal illumination output of 0.05 mW assuming 75% losses in the optical pathway. The counting frequency was typically 10–20 kHz. A 63× objective in combination with a 30 μ*M* diameter pinhole was used in all experiments. Fluorescence was selected by a OG515 long-pass filter. The FCS data were analyzed with nonlinear least-squares parametrization by the following equation [21]:

$$G(t) = 1 + \frac{(1 - F + Fe^{-\lambda_3 t})}{N}\left(\frac{1}{1 + t/\tau_d}\right)\left(\frac{1}{1 + t/a^2\tau_d}\right)^{1/2} \quad (1)$$

where N is the number of fluorescent particles in an uniaxial symmetric Gaussian volume element with radii of ω_{xy} and ω_z (e^{-2} point of the Gaussian beam). a is the ratio of ω_z to ω_{xy}. τ_d is the diffusion time (s), which is related to the translational diffusion constant D ($m^2\ s^{-1}$) by [21]

$$D = \frac{\omega^2}{4\tau_d} \quad (2)$$

F is the fraction of molecules in the triplet state and λ_3 is the characterizing singlet triplet transition in s^{-1}. The ratio $a=\omega_z/\omega_{xy}$ was obtained by measuring the diffusion of a 0.1 μ*M* solution of rhodamine in water under identical experimental conditions. The correlation function was fitted to Eq. (1) and $a=\omega_z/\omega_{xy}$ was fixed in the subequent analysis of the micellar data.

Time-correlated single-photon counting experiments were performed with a frequency-doubled (LBO frequency doubler) 1064-nm line of a mode-locked cw YLF laser (Coherent, Palo Alto, CA; Model Antares 76-YLF) synchronously pumping a cavity-dumped rhodamine 6G dye laser (Coherent; Model 701-2 CD) with an output at 295 nm after frequency doubling by a BBO crystal (Gsänger). The UV signal-illuminated sample (quartz 1×0.2-cm cuvette) and fluorescence were selected using a combination of WG 335-nm cut off and 348.8-nm interference (4.8-nm FWHM) filters. Details of the experimental setup are given elsewhere [13]. The TCSPC data were analyzed with the maximum entropy method (FAME, Maximum Entropy Data Consultants Ltd., Cambridge U.K.) [22]. First-order average lifetimes $\langle\tau\rangle$ were obtained by integration of the inverse Laplace transforms of the background corrected, deconvolved fluorescence decays:

$$\langle\tau\rangle = \frac{\sum_{i=1}^{N} \alpha_i\tau_i}{\sum_{i=1}^{N} \alpha_i} \quad (3)$$

where the summation is carried over all (N=100) τ_i values of an $\alpha(\tau)$ spectrum.

RESULTS

Fluorescence Correlation Spectroscopy

To determine the number of detergent molecules in Thesit micelles, the number of fluorescent particles N present in the volume element under observation was measured as function of molar ratio of the fluorescent label NBD-PE. Since the label is binomially distributed among the micelles, the probability $P_o(p)$ of finding a micelle without the probe at a molar ratio of p equals

$$P_o(p) = (1 - p)^S \quad (4)$$

where S is the size of the micelles in molecules. The probability $P_f(p)$ for a micelle to have one or more fluorescent lipid probes then equals $1-P_o(p)$. The number of fluorescent particles $N(p)$ at a labeling ratio of p present in the volume element of volume V is then

$$N(p) = \frac{CVA}{S}(1-(1-p)^S) \quad (5)$$

where C is the concentration of detergent molecules (M)

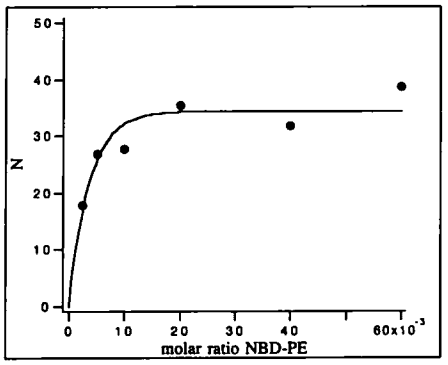

Fig. 2. Number of fluorescent particles counted in volume element versus molar ratio of NBD–PE in micelles. The detergent concentration used was 100 μ*M*. The solid line is the best fit of the data to Eq. (4).

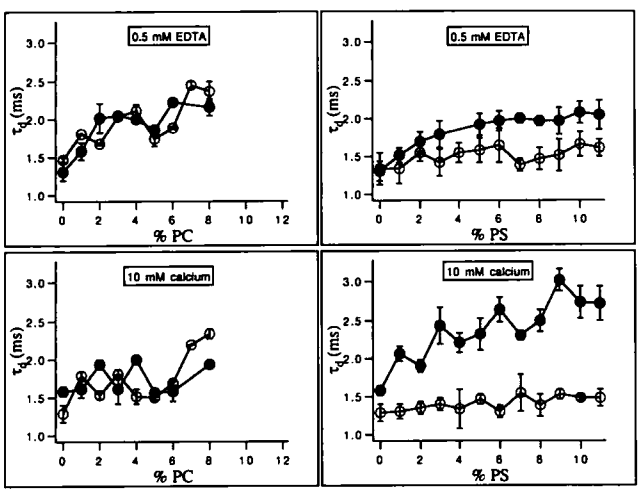

Fig. 3. Diffusion time of micelles as function of mole percent lipid. Left, τ_d vs mole percent PC; right, τ_d vs mole percent PS. Top, in the absence of calcium; Bottom, In the presence of 10 m*M* calcium. Open symbols, τ_d vs mole percent lipid in the absence of PKC; filled symbols, τ_d vs mole percent lipid in the presence of 0.75 μ*M* PKC.

and *A* is Avogadro's number. In Fig. 2 the number of counted labeled micelles (*N*) as function of the molar ratio NBD-PE (*p*) at 100 μ*M* thesit is presented. The same figure contains the fit of the data to Eq. (5) with two adjustable parameters (*S, V*). From this analysis we obtained a value of 272 for *S* and 0.15 fl for *V*, which is in fair agreement with the value of 0.24 fl as found from the diffusion of rhodamine 6G in the same volume element [21]. The parameter *S* enables us to estimate the concentration of micelles to be 0.37 μ*M*. The interaction of PKC with PS and PC was studied in the presence and absence of an excess of calcium (Fig. 3). In these experiments the protein concentration was 0.75 μ*M*, which is a twofold excess over the micelles. As can be seen in the two left panels in Fig. 3, there is no influence of PKC on the diffusion time of PC-loaded micelles. A gradual increase in diffusion times with the percentage content of PC was observed for the micelles alone. The reason for this is unclear and was not observed for PS loaded micelles. One possible reason is that PC micelles experience a larger microviscosity due to the presence of a sphere of "structured" water. Above 8% PC the mixed micelles were unstable, as apparent from the presence of large aggregates of lipids with large diffusion times (data not shown). A PKC-dependent increase in diffusion times could be detected when PS was present in the micelles. Surprisingly, the interaction of PKC with PS loaded micelles occurred also in the absence of calcium. When calcium was present in the buffer the increase in the diffusion times at a high molar ratio of PS was two to three times as large as when calcium was absent in the buffer. This relatively larger increase in diffusion times in the presence of calcium can have three origins: (i) Micellar aggregates are formed in the presence of calcium and PKC, (ii) the stoichiometry of binding is different in the presence and absence of calcium, and (iii) the dissociation constant of the PKC micelle complex is much higher in the absence of calcium. The first reason can be excluded since we did not find evidence of a PS- or calcium-dependent decrease of *N*, which is expected when the aggregation number of the micelles is changed (data not shown). The discrimination between the second and the third possibility is more difficult. The calculated diffusion constant for PKC at 2.4 cp obtained from the Stokes–Einstein relation assuming that it is spherical is $3.1 \cdot 10^{-7}$ cm^2 s^{-1}. For rhodamine in water we obtained a diffusion time of 45 μs at 63× magnification and 30 μ*M* pinhole. By means of Eq. (2) we obtained a value for ω^2 of $5.04 \cdot 10^{-10}$ cm^2, which then gives us the diffusion time of PKC of 0.39 ms. Since the diffusion time is proportional to the radius of the measured particle, this value for PKC should be close to the maximal increase in diffusion time due to the association of a single molecule to mixed micelles (when it becomes an elongated particle). Inspection of Fig. 3 shows that this is approximately the increase in diffusion time due to protein at a high PS content of the micelles. In the presence of calcium the increase in the diffusion time due to protein is much larger than theoretically possible for the association of a single molecule of PKC per micelle. In this case the most likely amount of protein molecules per micelle is two, since a twofold excess of protein was used over the micelles. To investigate further the calcium dependence of PKC–PS interactions, we performed a titration of micelles containing 10% PS with increasing amounts of PKC in the presence

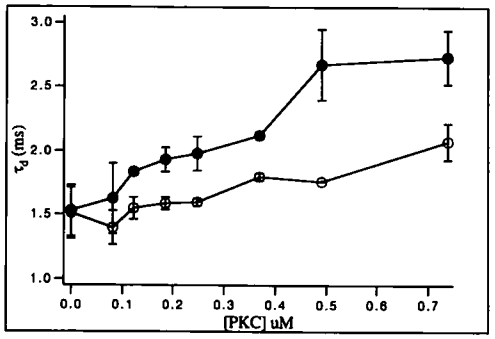

Fig. 4. Diffusion time of micelles loaded with 10% PS as a function of PKC concentration. Open symbols, absence of calcium; filled symbols, presence of 10 m*M* calcium.

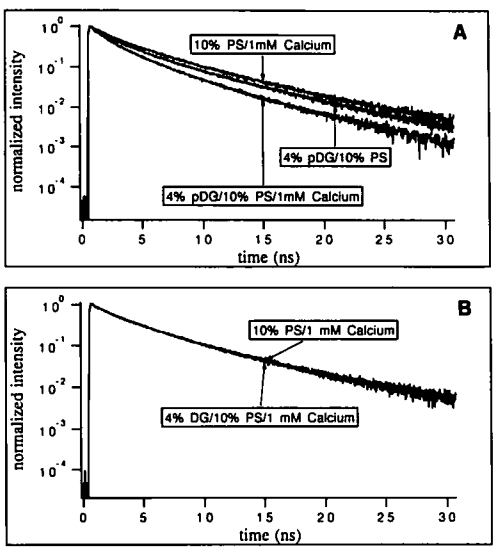

Fig. 5. Normalized fluorescence decays of PKC in the presence and absence of pDG (A) and DG (B), respectively. The composition of the mixed micellar systems was 100 μ*M* thesit and 10% PS in all the experiments.

and absence of 10 m*M* calcium (Fig. 4). The diffusion time of micelles increases at a low protein concentration in the presence of 10 mM calcium. In the absence of calcium the diffusion time does not significantly change below 0.3 μ*M* protein, above which the diffusion time gradually increases due to association of the protein with the micelles. Without modelling the data, it is then likely that the affinity of PKC for PS-loaded micelles is much lower in the absence of calcium.

Time-Resolved Fluorescence

The interaction of PKC with lipid cofactors was studied with an excess of micelles over protein in the presence of 1 m*M* calcium. In this way a single binding site for the lipid cofactor is present per protein–micelle complex. By excitation at 295 nm and detection of fluorescence at 340 nm, only the tryptophans in PKC are monitored, leaving out the micelles which did not form a complex with protein. Since the micelles have a limited size of *S* surfactant molecules, the number of lipid molecules (n) per micelle at a labeling ratio $p=n/S$ is binomially distributed. The fraction of complexed protein molecules $C(p)$ at a labeling ratio of p is [13,14]

$$C(p) = \sum_{n=1}^{S}\binom{S}{n}p^n(1-p)^{S-n}\frac{K+n+1-\sqrt{(K+n+1)^2-4n}}{2} \quad (6)$$

where K is the dissociation constant of the protein–lipid complex in the molecule. $C(p)$ can be estimated from the average lifetime $\langle\tau\rangle$ of the tryptophans in PKC at a certain labeling ratio p of the micelles [13,14]:

$$\langle\tau\rangle = [1-C(p)]\langle\tau_{pkc}\rangle + C(p)\langle\tau_c\rangle \quad (7)$$

where $\langle\tau_{pkc}\rangle$ is the average lifetime of uncomplexed PKC and $\langle\tau_c\rangle$ is the average lifetime of complexed PKC. The observable $\langle\tau\rangle$ is fitted as a function of p to Eq. (7) with four adjustable parameters: S, K, $\langle\tau_{pkc}\rangle$, and $\langle\tau_c\rangle$. The parameters $\langle\tau_{pkc}\rangle$ and $\langle\tau_c\rangle$ are solved by linear least-squares analysis for any given pair of S and K. The nonlinear parameters S and K are solved by nonlinear least-square routines. By plotting the sum of squares of residuals (SSQ) of the lipid binding data as function of the parameters S and K, it is inferred that these parameters are highly correlated and that the minimum of SSQ is not well defined [13]. Fortunately we obtained S from correlation spectroscopy experiments as outlined above. This leaves only K as a nonlinear parameter in the model. In Fig. 5A the effect of calcium on the interaction of 4% pDG loaded in mixed micelles of 10% PS with PKC is shown. The faster fluorescence decay demonstrates that the presence of micelles loaded with PS and labeled DG give rise to energy transfer from PKC to the pyrene in the presence of calcium. This decrease in average lifetime is due solely to energy transfer from tryptophans in PKC to the pyrene moiety in DG as demonstrated in Fig. 5B, where no effect on the fluorescence decay is observed when 4% DG is incorporated in the micelles. A similar situation was observed for the other investigated lipids. The average fluorescence lifetime of the tryptophans in PKC as function of the fraction of labeled lipids in the micelles is presented in Fig. 6 together with the optimized fits to Eq. (7). As expected, $\langle\tau\rangle$ does not decrease with an increase in the molar fraction of pyrene-labeled phosphatidylcholine

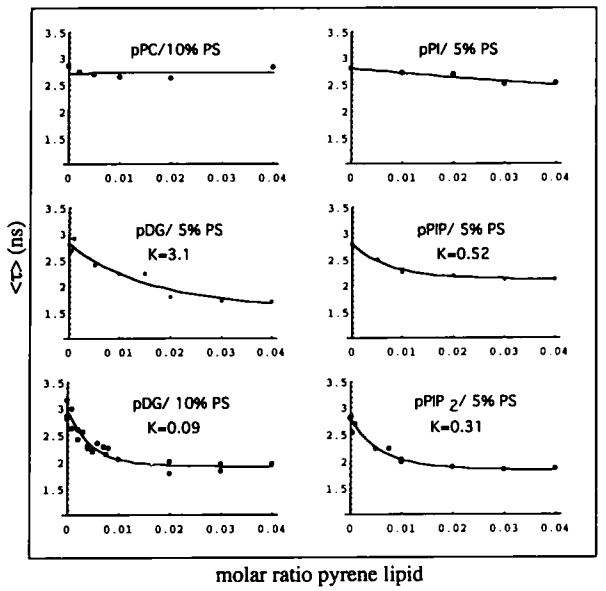

Fig. 6. Average fluorescence lifetime $\langle\tau\rangle$ vs molar ratio of pyrene/lipid. In all the experiments the detergent concentration was 100 μM and 1 mM calcium was present in the buffer. The solid lines are the best fit of the data to Eq. (7). The values of the obtained dissociation constant K are given where applicable. The symbols are explained in the text.

(pPC) since this lipid does not bind to PKC. It is, of course, expected that at higher molar ratios of pPC, nonspecific energy transfer will take place to the random distribution of pyrenes in the micelles which do not interact with PKC directly. In contrast, the average lifetime clearly drops with an increase in the molar fraction of pDG, which shows that this is a specific interaction. In Fig. 6 it can also be seen that the binding constant for pDG decreases at a higher mole fraction of PS, which is indicative of a cooperativity between the binding of PS and DG. It is essential in this case to show that all PKC molecules are bound to micelles under these conditions; otherwise the difference in K could originate from a DG-induced translocation of the protein. This can be done by titrating the protein with micelles as demonstrated in another paper [14]. PKC also interacts with precursor molecules of DG, namely, the phosphoinositides as shown on the right side in Fig. 6 [14]. The affinity increases as more phosphate groups are present on the phosphoinositide. The affinity of $pPIP_2$ for PKC at 5% PS is higher than that of pDG, which puts in question whether the PLC-dependent hydrolysis of PIP2 is really the mechanism to activate PKC on the lipid interface. Displacement studies with pDG and pPIP2 provided evidence that these cofactors cannot bind simultaneously to the protein [14]. More importantly, it was also found that not only DG can activate the protein, but also PIP_2 [23].

DISCUSSION

Diffusion monitored by FCS and resonance energy transfer detected by TCSPC provide sensitive means to study the interaction of proteins with lipid aggregates. With the former technique the interaction between the protein and the whole lipid aggregate can be studied, whereas with the latter technique specific interactions within the micelle can be quantified. In this sense both techniques are complementary in their information content. This is also exemplified by the fact that, in order to obtain dissociation constants for the lipid cofactors and PKC (TCSPC data), the micellar size had to be measured by FCS. The use of a micellar system instead of vesicles can be thought of as inconvenient owing to the additional parameter S; the advantage is that one does not need to know the concentration of protein exactly to determine K as long as it is smaller than the micellar concentration. The largest disadvantage of a micellar system is that it does not mimic the biological bilayer structure with its physicochemical properties, and care should be exerted to extrapolate results obtained with this system to the functioning of proteins *in vivo*. A simple question which can be answered with this system is whether or not a lipid specifically interacts with a protein. In this respect we confirmed by FCS that PKC interacts with PS but not with PC. Striking was that the protein associates with PS-loaded micelles in the absence of calcium, although with a lower affinity. A similar finding was reported by another group using vesicles instead of micelles [19]. The question is, then, if *in vivo* the protein is already associated with the membrane before it becomes activated.

With time-resolved fluorescence we found that DG and the precursor molecules PIP_2 and PIP bind with a high affinity to PKC but only DG and PIP_2 could activate the protein [14]. The PLC-dependent hydrolysis of PIP_2 *in vivo* is then not necessary to activate the protein. Other pathways can then also function as "switches" for the activity of PKC. For example, phosphorylation of PIP by PIP-kinase to PIP_2 or hydrolysis of PC to DG by a specific PLC could, in combination with influx of extracellular calcium, be another route which activates the protein. In this way, the cytosolic (calcium) and membrane (DG, PIP_2) messages are uncoupled, which could lead to more diverse signaling pathways to acti-

vate this protein, which is involved in multiple signal processing in the cell.

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