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The interaction of pyrene labeled diacylglycerol with protein kinase C in mixed micelles

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Abstract

The binding of protein kinase C (PKC) to pyrene-labeled diacylglycerol (pDG) has been studied in a mixed micellar system by monitoring resonance energy transfer from excited tryptophans to pyrene with time-correlated single photon counting. The average lifetime of the excited state of the tryptophans in PKC showed a clear dependence on the mole percentage pDG in micelles in contrast with pyrene-labeled phosphatidylcholine (pPC). The binding data has been analyzed to a simple model which encompasses the size of the micelles and the binding constant of the pDG–PKC complex. From our data, though, these quantities cannot be determined independently. If we have no size information on the micelles we can determine a lower boundary of this quantity compatible with the data. When the micellar size is known, a binding constant for the DG–PKC complex can be extracted. The presented analytical approach can be applied to other systems in which lipid–protein interactions must be quantified.

Keywords: Protein kinase C; Pyrene; Micelles; Lipid–protein interaction; Fluorescence resonance energy transfer

1. Introduction

The serine/threonine protein kinase C (PKC) family has a key role in the phosphoinositide signal transduction pathways [1–6] and is the major receptor for tumor promoting phorbol esters [7,8]. Nine isoenzymes have been identified so far [9–13] with tissue specific expression [5]. The 80 kDa protein isolated from rat brain consists of three major species [14] with similar physico-chemical properties [15] to which we will refer as a single entity "PKC". PKC activation requires a synergistic action of elevated cytosolic calcium levels and the lipid cofactor diacylglycerol (DG) [2,3]. The latter is generated by hydrolysis of phosphoinositidebiphosphate (PIP_2) or phosphatidylcholine (PC) through action of specific phospholipases C [6,16–19]. Although some controversy exists in literature (reviewed in [20]), it is thought that elevated calcium levels cause a translocation of PKC from the cytosol to the membrane where it becomes activated by interaction with DG. The interaction with the plasma membrane has an absolute requirement for negatively charged phospholipids such as phosphatidylserine [21,22]. For a more elaborate dis-

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cussion on the extensive biochemistry of the protein we refer the reader to a recent review [23].

The interaction of PKC with its lipid cofactors has been extensively studied by optical techniques such as fluorescence [15,24–27], light scattering [28] and circular dichroism (CD) [29,30]. In all these studies the interaction is measured indirectly by monitoring dipole–dipole interaction with inert lipids by fluorescence spectroscopy (resonance energy transfer to NBD-PE), the increase of particle size due to interaction with the protein by light scattering techniques or changes in optical activity of chiral molecules with CD.

In this paper we report on the interaction of PKC with the fluorescently labeled cofactor diacylglycerol (pDG) in a mixed micellar system [21]. The advantage of this approach is that the biochemical compounds of interest (PKC and labeled cofactor) interact directly by a dipole coupling mechanism (resonance energy transfer). To this purpose we have synthesized a fluorescent DG (pDG) by attaching a fluorescent pyrenedecanoic acid to the *sn*-2 position of the lipid glycerol. The fluorescent pyrene moiety is an excellent energy acceptor of singlet excited tryptophans with an approximate critical transfer distance of 2.7 nm [31]. The efficiency of this short range interaction is dependent on the sixth power of the distance between the interacting groups which provides a sensitive tool to study binding between lipid cofactor and PKC. In steady-state fluorescence spectroscopy, such as has been exploited by several laboratories [15,24–27], energy transfer is monitored by the decrease of the donor (tryptophans in PKC) emission or the increase of sensitized acceptor (labeled lipids) emission. 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. In addition, (1) the dynamic range is much larger than its steady-state counterpart which makes it a much more sensitive technique and (2) eventual conformational changes in the protein can be more readily detected. Our goal is to obtain quantitative information on the binding constant between PKC and the cofactor DG in a mixed micellar system.

2. Materials and methods

PKC was isolated from 30 Wistar rat brains according to the procedure described by Huang et al. [14]. The yield of the isolation was around 500 μg with a specific activity of 300 units/mg at 20°C. The protein was > 95% pure as judged by silver stained SDS-PAGE. Activity was measured by phosphorylation of calf thymus histone IIIS (Sigma) with $\gamma^{32}P_i$-ATP (Amersham) for 10 min at 20°C [32]. One unit is defined as 1 nmol incorporated P_i per min. The enzyme preparations were stored in 200 μl aliquots (60 μg/ml) at −70°C in 20 m*M* Tris buffer, pH 7.5, 100 m*M* NaCl, 0.5 m*M* EDTA, 0.5 m*M* EGTA, 25% glycerol (Merck fluorescent microscopy grade), 1 m*M* β-mercaptoethanol, 100 μ*M* PMSF. All buffers were made with nanopure water prepared on a millipore water purification system. Bovine brain phosphatidylserine (PS), dioleoylphosphatidylcholine (PC) and diacylglycerol (DG) are from Sigma Chemical Co. (St. Louis, MO). Fluorescently labeled DG (pDG) was obtained by phospholipase C catalyzed hydrolysis of pyrene labeled PC (pPC) as described elsewhere [31]. Stock solutions of mixed micelles were prepared as follows: lipids dissolved in chloroform were mixed in a fixed quantity in a test tube. A film of these lipids was created by evaporating the chloroform 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. In all the described experiments the content of phosphatidylserine in the micelles was 10% (molar) with a varying mole percentage of pDG. Of this stock solution 15 μl was added to a reaction mixture containing 105 μl 20 m*M* Tris pH 7.5, 100 m*M* NaCl and 30 μl PKC giving final concentrations of 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 and 0.16 μ*M* PKC. The reaction mixture was allowed to equilibrate for 15 min at 20°C before a measurement.

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 590 nm. The vertically polarized output was rotated to horizontally polarized light by a variable wave plate (New Focus 5540) after which it was frequency doubled by a BBO crystal (Gsänger) to 295 nm with vertical polarization. The repetition frequency of the pulses was 951 kHz after the cavity dumper with a FWHM of 4 ps and tens of pJ pulse energy. Part of the red output of the dye laser was collected onto a fast PIN photo diode connected to a constant fraction discriminator (CFD, Tennelec, model TC 864) to trigger the stop signal to the time to amplitude converter (TAC, Tennelec model TC 864). The UV signal illuminated sample (quartz 1×0.2 cm cuvet) and polarized fluorescence was selected using a combination of WG 335 cut-off and 348.8-nm interference (4.8 nm FWHM) filters and a computer controlled motor driven sheet type polarizer (Polaroid NHP'B). The start signal for the TAC was triggered by the arrival of a photon onto the microchannel plate (MCP, Hamamatsu, 1645 U) connected to a wide band amplifier (Hewlett-Packard 8447F) and let through a channel of the CFD. The analog signal of the TAC was digitized by a analog to digital converter (Nuclear Data, ND582) and a count was registered in the corresponding channel (ch) of the multichannel analyzer (Nuclear Data ND66) set at 30 ps/ch over 1024 channels. The counting frequency was 30 kHz. Measurements consisted of 10 sequences of measuring 10 s alternately in parallel and perpendicular polarization directly followed by a 4-sequence measurement of the background. A reference compound (PTF in ethanol, 1.06 ns [33]) was used to obtain the instrumental response function before and after a sample measurement at 3 sequences of 10 s. Part of the experiments were performed at the Karolinska Institute at the Department of Medical Biophysics on a time-correlated single photon counting setup with a modelocked argon ion laser and synchronously pumped frequency doubled rhodamine 6G dye laser as excitation source as extensively described elsewhere [34]. Good agreement exists between the results obtained with the two instruments.

The data were analyzed with the commercially available maximum entropy method (FAME, Maximum Entropy Data Consultants Ltd., Cambridge UK). With this analysis one obtains a transformation of the fluorescence decay from time-space to lifetime-space (inverse Laplace transform) [35]. First-order average lifetimes $\langle\tau\rangle$ were obtained by integration of the inverse Laplace transforms of the background corrected, deconvolved fluorescence decays:

$$\langle\tau\rangle = \sum_{i=1}^{N} \alpha_i\tau_i / \sum_{i=1}^{N} \alpha_i \qquad (1)$$

where the summation is carried out over all (N = 100) τ_i values of an $\alpha(\tau)$ spectrum. This parameter is proportional to the integral of the decay function over time.

From this data the binding of PKC to fluorescently labeled DG was quantified as follows. Let us assume that the binding within a micelle is governed by a dissociation rate constant k^- (sec^{-1}) and a rate of association k^+ ($molecule^{-1}$ sec^{-1}). As long as there is at most one PKC molecule per micelle ([PKC] $\ll$ [micelle]) and one ligand (L) we have:

$$PKC + L \underset{k^-}{\overset{k^+}{\rightleftharpoons}} C$$

and at equilibrium:

$$k^+[L][PKC] = k^-[C] \qquad (2)$$

where [C] is the "concentration" of complex (of dimension molecules). Since the total PKC "concentration" ([PKC] + [C]) corresponds to unity, C is a number between zero and one expressing the probability of finding the complex in time. Averaged over an ensemble of many micelles the equilibrium constant K (k^-/k^+) is:

$$K = (1 - C)^2/C \qquad (3)$$

as [L] and [PKC] are unity, providing $C \neq 0$. If we permit several ligands (n) in the micelle the dissociation constant is:

$$K = (1 - C)(n - C)/C \qquad (4)$$

Solving the quadratic equation for $C(n)$, one obtains:

$$C(n) = \frac{K + n + 1 - \sqrt{(K + n + 1)^2 - 4n}}{2} \quad (5)$$

Since the mixed micelles have a limited size of S surfactant molecules the variable n is distributed according to a binomial distribution:

$$P(n) = \binom{S}{n} p^n (1-p)^{S-n} \quad (6)$$

where $P(n)$ is the probability of finding n cofactor molecules at a labeling ratio of $p = n/S$. The total amount of complexed molecules (C_{tot}) at a given labeling ratio p is then:

$$C_{tot} = \sum_{n=1}^{S} P(n)C(n) \quad (7)$$

where the summation is initiated at $n = 1$ since $C(0) = 0$. With the average lifetime $\langle \tau \rangle$ obtained at a certain labeling ratio p one can estimate C_{tot}:

$$\langle \tau \rangle = (1 - C_{tot})\langle \tau_{pkc} \rangle + C_{tot}\langle \tau_c \rangle \quad (8)$$

where $\langle \tau_{pkc} \rangle$ is the average lifetime of uncomplexed PKC and $\langle \tau_c \rangle$ is the average lifetime of complexed PKC. In practice the observable $\langle \tau \rangle$ as a function of p is fitted to the rearranged equations (7) and (8) to obtain the parameters a, b, K and S:

$$\langle \tau \rangle = a - b \sum_{n=1}^{S} P(n)C(n) \quad (9)$$

where the parameters a and b equal $\langle \tau_{pkc} \rangle$ and $\langle \tau_{pkc} \rangle - \langle \tau_c \rangle$, respectively and $P(n)$ and $C(n)$ are determined from K and S by eqs. (5) and (6). The parameters a and b are solved by linear least squares analysis for any given pair of S and K [36]. Thus equation (9) has only two non-linear parameters K (equilibrium constant in molecule) and S (size of micelle in units of number of surfactant molecules) left which can be obtained by fitting the data by non-linear least squares routines. The data was fitted to eq. 9 with non-linear least squares routine supplied with the program Mathematica [37] based on the Marquardt algoritm [38].

3. Results

The effect of 4% pDG loaded in mixed micelles of 10% PS on the heterogeneous tryptophan fluorescence decay of rat brain PKC is shown Fig. 1A. 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. Unlabeled DG had no effect on the fluorescence decay of PKC which shows that it is energy transfer to the pyrene label and not the presence of the cofactor which reduces the lifetime (Fig. 1B).

The average lifetime $\langle \tau \rangle$ is a parameter which can be related to the efficiency of energy transfer [39] and is as such a proper parameter to quantify interaction between pDG and PKC (Fig. 2). Calcium causes a slight decrease of the average

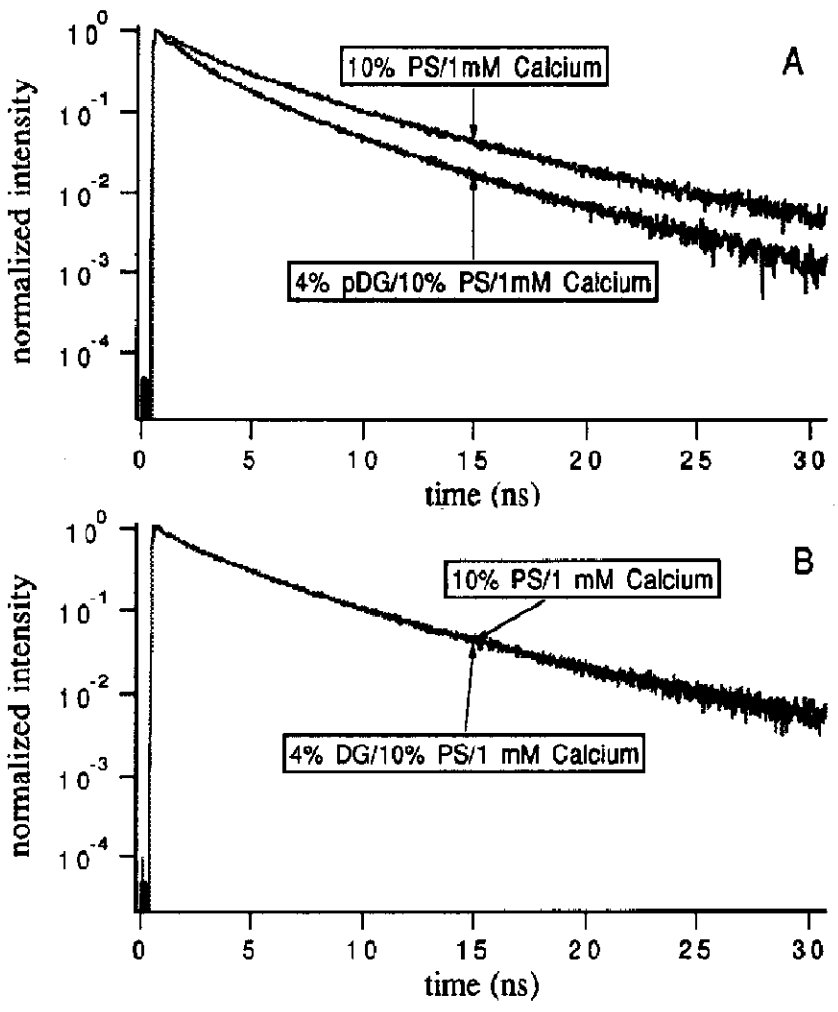

Fig. 1. Normalized fluorescence decays of PKC in the presence and absence of pDG (A) and DG (B) respectively. The composition of the mixed micellar systems was 100 μM Thesit and 10 mol% PS in all the experiments.

fluorescence lifetime of PKC (panel A). The calcium induced decrease in average lifetime is further enhanced in the presence of micelles containing 10% PS (panel B). This effect can be accounted for by a conformational change of the protein upon binding to negatively charged phospholipids. Such a change in the conformation of PKC upon binding to PS has been postulated before [20,32,40]. It is remarkable that the sole presence of PS loaded micelles causes hardly any decrease of $\langle\tau\rangle$ whereas the mixture of calcium and the chelator EDTA together cause a substantial decrease in $\langle\tau\rangle$ when PS is present (panel A and B). Since EDTA is a calcium chelator this would indicate an irreversible conformational change when PKC binds to PS in the presence of calcium. By comparison of panel C and D it can be seen that addition of calcium causes a drastic decrease in $\langle\tau\rangle$ only when DG is labeled with the pyrene in agreement with the fluorescence decays as depicted in Fig. 1A. The binding of pDG seems to be partly reversible upon addition of EDTA. Correction for the decrease in lifetime due to above mentioned conformational change yields an almost 80% recovery of the original lifetime which shows that binding of pDG as a result of calcium is almost completely reversible in this system.

The normalized inverse Laplace transform of the fluorescence decays of PKC as obtained by the MEM analyses are shown in Fig. 3. The heterogeneous decay gives rise to 4 lifetime classes in a system containing 1 mM calcium and micelles loaded with 10% PS. The three major peaks shift to shorter lifetimes in the presence of 4% pDG whereas the peak at approximately 0.1 ns is practically invariant to the presence of acceptor probe. The shift of the major lifetime classes in the presence of pDG shows that the majority of the tryptophans is able to interact

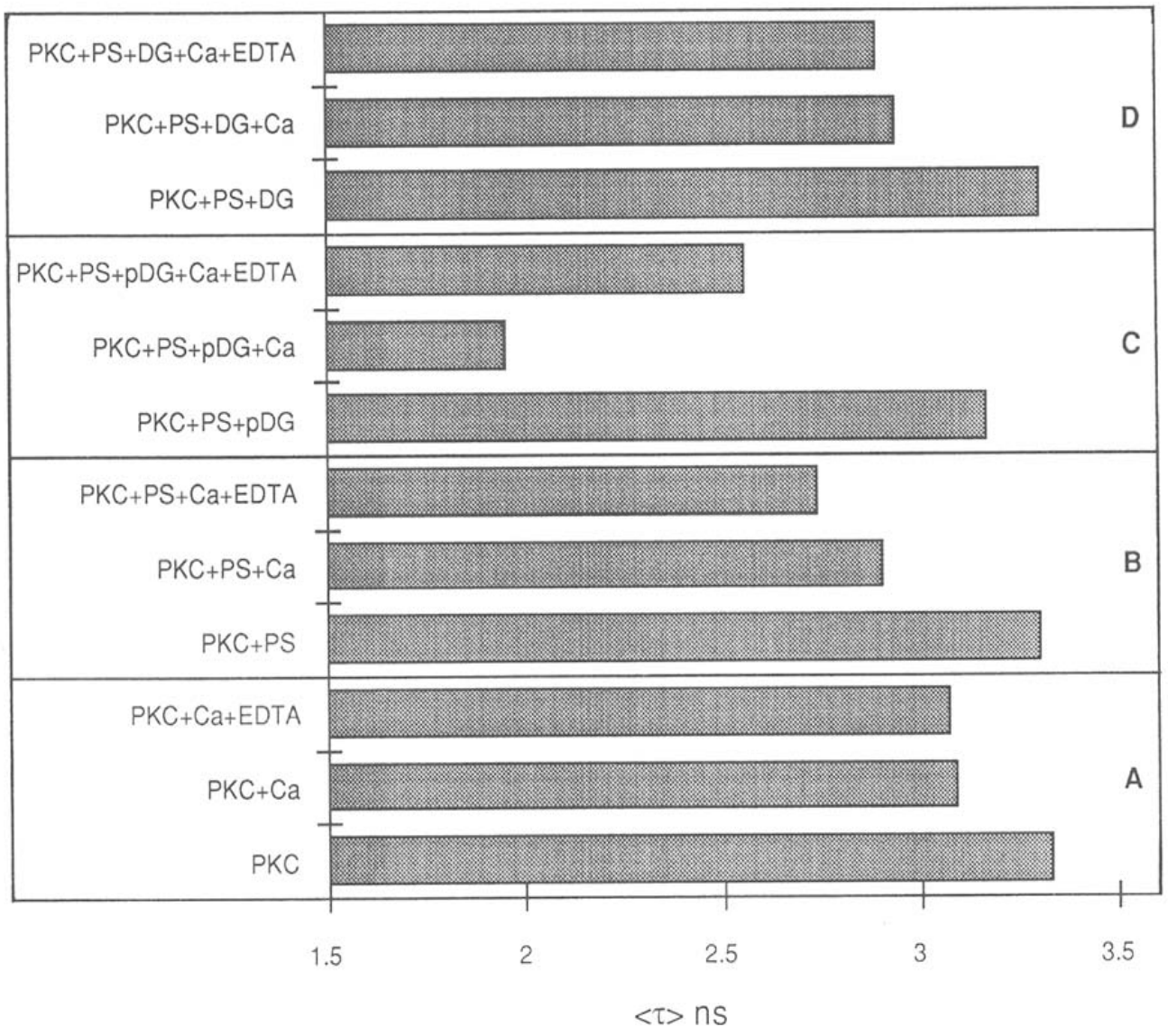

Fig. 2. First order average fluorescence lifetimes of tryptophan residues in PKC under different conditions. Panel A: effect of 1 mM calcium. Panel B: effect of 1 mM calcium in the presence of 100 μM thesit micelles loaded with 10 mol% PS. Panel C: effect of 4 mol% pDG in 100 μM Thesit micelles loaded with 10 mol% PS. Panel D: effect of 4 mol% DG in 100 μM Thesit micelles loaded with 10 mol% PS. In all the panels the EDTA concentration was 10 mM.

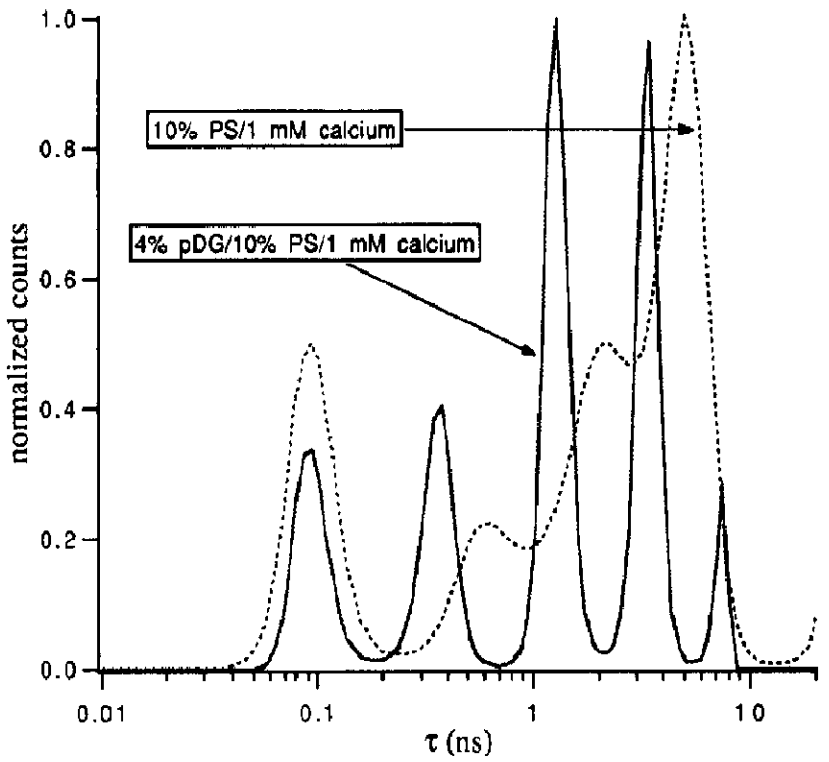

Fig. 3. Normalized inverse Laplace transforms of the fluorescence decays of PKC in a buffer containing 100 mM thesit micelles loaded with 10 mol% PS in the presence and absence of 4 mol% pDG. 100 equally spaced τ values on a logarithmic scale between 0.01 ns and 20 ns were used in the reconstruction of the lifetime spectra by the MEM method.

through dipole–dipole coupling with the pyrene moiety on the DG fatty acid chain. The complexity of a system containing eight tryptophans does not allow for a detailed photophysical interpretation of lifetime classes. Instead, an overall property such as the average lifetime is a more appropriate parameter to monitor binding of the protein to lipids since one is observing an average change upon binding and not an absolute quantity. The average lifetime $\langle\tau\rangle$ of PKC as function of mole percent pDG is presented in Fig. 4. The continuous decrease of $\langle\tau\rangle$ which saturates at above 2 mol% pDG shows that binding of pDG can be quantified with the average lifetime of excited tryptophans in PKC. In a control experiment, the average lifetime of PKC versus mole percentage of the inert lipid pPC was measured and is plotted in the same figure. Clearly no drastic decrease in $\langle\tau\rangle$ can be observed upon increase of mole percentage pPC which shows that the binding of pDG is specific.

By plotting the sum of squares of residuals (SSQ) of the pDG $\langle\tau\rangle$ data as function of the parameters size (S) and the binding constant (K) (see Fig. 5), it is inferred that these parameters are highly correlated and that the minimum of

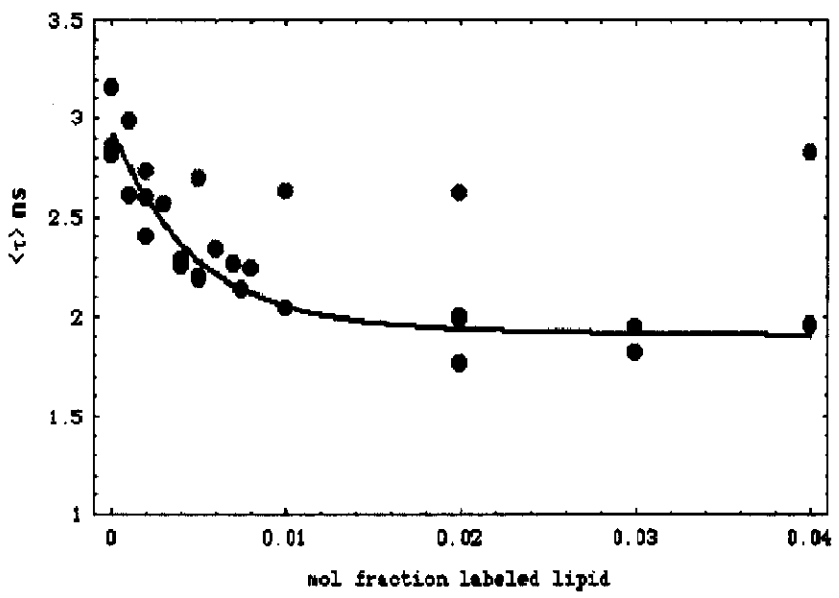

Fig. 4. Dependence of average lifetime τ of PKC on mole fraction pDG (black circles) or pPC (gray circles) in 100 mM Thesit micelles containing 10 mol% PS. The solid curve is the best fit of eq. (9) to the pDG data by assuming a micellar size of 330 units.

SSQ is not well defined. If one assumes that every PKC molecule bound to a micelle containing pDG is occupied with an acceptor probe ($K = 0$) one can estimate a minimal size of the micelles. This search is presented in Fig. 6 from which it can be deduced that micelles are bigger than 200 detergent molecules. From fluorescence correlation spectroscopy on NBD-PE labeled micelles we have estimated their size to be 330 surfactant molecules (unpublished results). This parameter can be fixed in the fit of the $\langle\tau\rangle$ vs.% pDG data to obtain a defined minimum of SSQ for K (Fig. 7). The corresponding value of 0.15

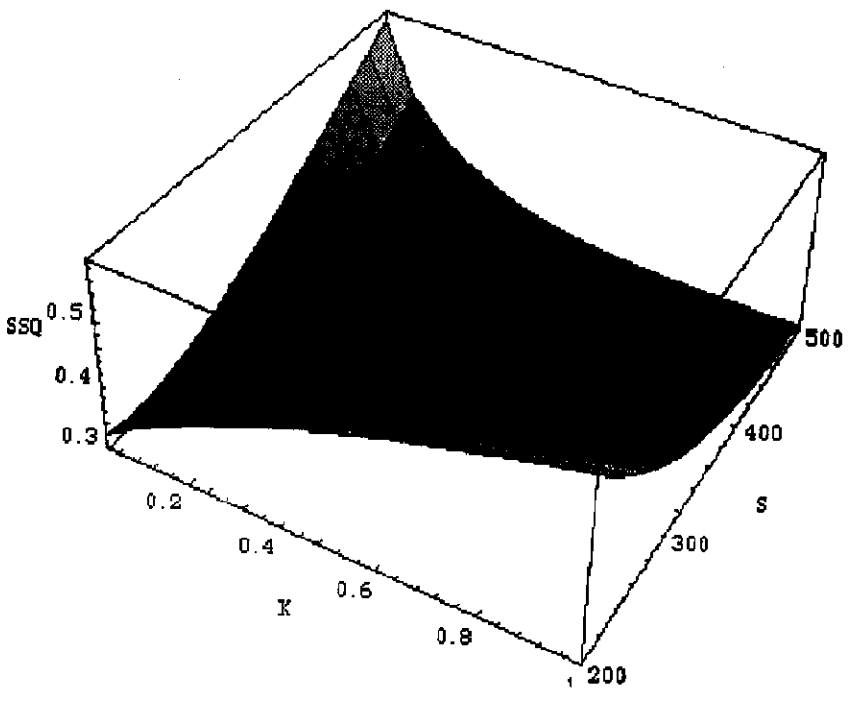

Fig. 5. SSQ of the mole percentage pDG vs. $\langle\tau\rangle$ data as a function of the non-linear parameters S and K.

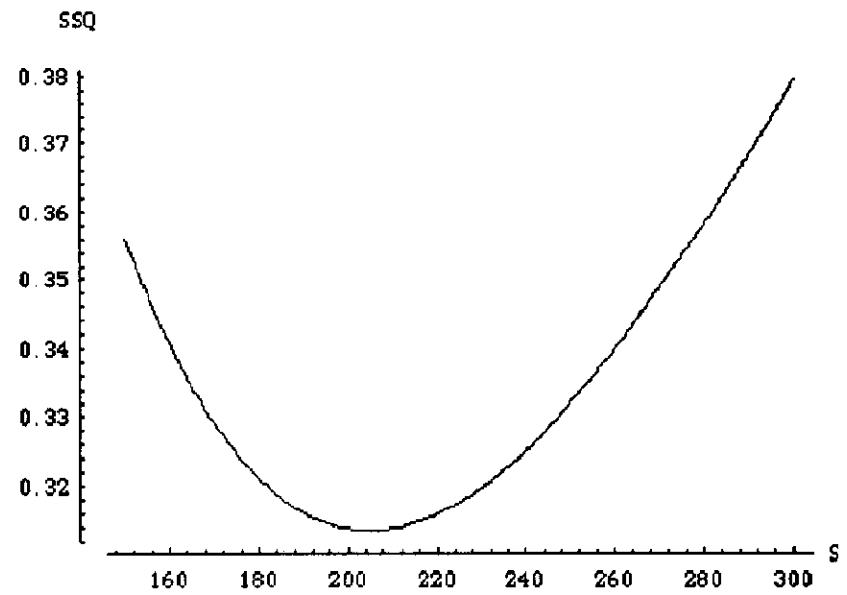

Fig. 6. SSQ of the mole percentage pDG vs. $\langle\tau\rangle$ data as function of the parameter S in which K was fixed to a value of 0.

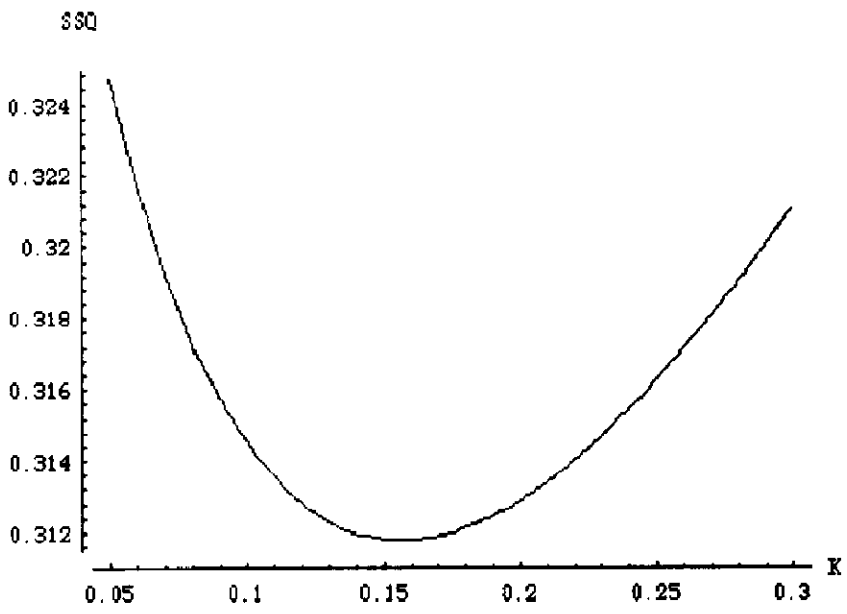

Fig. 7. SSQ of the mole percentage pDG vs. $\langle\tau\rangle$ data as function of the parameter K in which S was fixed to a value of 330.

molecules for K demonstrates that the lipid cofactor has a high affinity for the protein under the experimental conditions.

In order to verify that DG and pDG bind on the same site on the protein, PKC was incubated with micelles containing 2 mol% pDG and a variable mol% DG (0–6%). The increase of $\langle\tau\rangle$ with the increase in ratio of DG/pDG shows that both molecules compete for the same binding site (Fig. 8).

4. Discussion

In this study the interaction of pyrene labeled DG with PKC in the presence of calcium was quantified by time-resolved fluorescence spectroscopy. The *a priori* accepted binding model (see Section 2) is probably oversimplified in the fact that it assumes a single binding site, no cooperativity and reversible binding of pDG. In a more elaborate study using a similar interaction model as presented here the number of binding sites for phosphoinositides was estimated to be smaller or equal to two [31]. Hannun et al. [21] showed in a mixed micellar system that full activity of PKC was achieved when a single DG molecule was bound to PKC. It is then reasonable to assume that there is a single binding site for DG on PKC. Were binding of pDG irreversible in the presence of calcium, one should find after long times that every PKC molecule attached to the micelles would contain a labeled DG. This time dependence of increased occupancy was not found (data not shown). The binding could be irreversible when the size of the micelles as estimated with correlation spectroscopy (≈ 330) was overestimated. In this case,

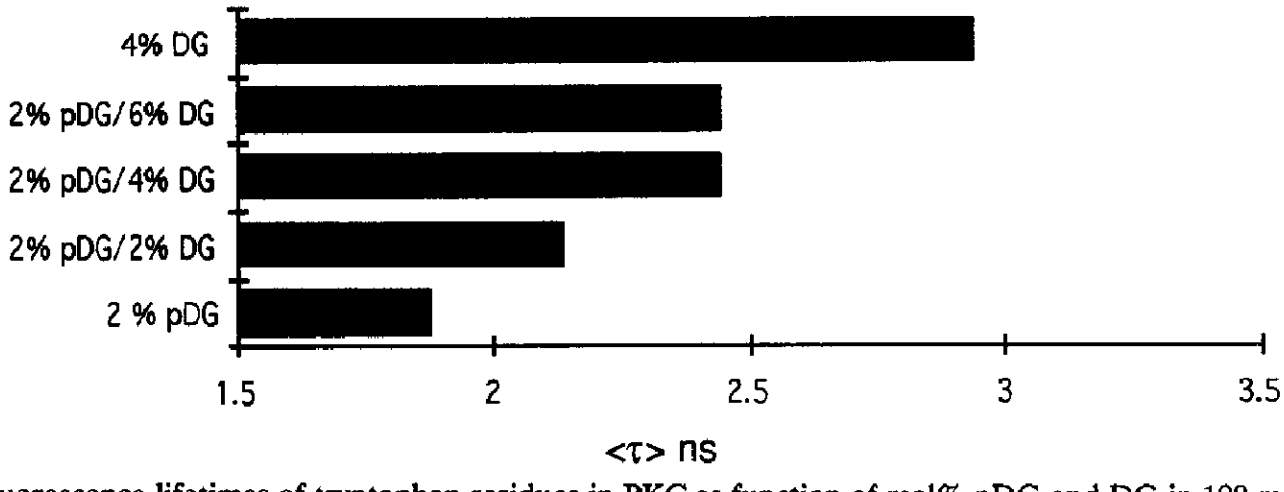

Fig. 8. Average fluorescence lifetimes of tryptophan residues in PKC as function of mol% pDG and DG in 100 mM Thesit micelles containing 10 mol% PS.

the decrease of $\langle\tau\rangle$ as function of mole percentage pDG is solely due to the binomial distribution of probe over the micelles owing to their finite size. This situation is unlikely since it was found that at 5 mol% PS the dissociation constant (K) of pDG for PKC is on the order of 3 [31] which shows that the binding affinity of pDG for PKC is strongly dependent on PS. Lee and Bell [41] also found that the DG induced activity of PKC was strongly dependent on the PS content of mixed micelles.

Titration of a micellar PKC complex containing 2 mol% pDG with DG (0–6%) did result in an increase of $\langle\tau\rangle$ which shows that both molecules compete for the same binding site. The $\langle\tau\rangle$ is not restored to the value of 2.78 ns at a molar ratio of 3 for DG/pDG but to 2.45 ns. This can be explained by a higher affinity of pDG as compared to DG for PKC. Due to the observed saturation of the displacement curve it is in our opinion more likely that the displaced pDG remains in the vicinity of PKC where it is still within the Förster radius of the donor molecules (tryptophans). This proximity hypothesis can be sustained by two effects: (1) the small size of the micelles gives rise to an average distance between "free" pDG and PKC sufficient for energy transfer, and (2) clusters of PS are formed in which (p)DG molecules preferentially partition and thus interact with PKC via energy transfer. From our data we cannot conclude whether one of the above explanations is the predominant factor causing the low value of $\langle\tau\rangle$ at high DG/pDG ratio.

Although the use of a micellar system instead of for example 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 PKC exactly to determine K as long as it is smaller than the micellar concentration. From a biochemical point of view the micellar system does not mimic the biological bilayer structure with its specific physicochemical properties and one can not extrapolate results obtained with this system to the functioning of PKC *in vivo*. In this context it is for example reported that DG causes membrane bilayer perturbations which can activate PKC [20]. Despite this fact, interactions between lipid cofactors and the kinase can be demonstrated and compared in a relatively simple experimental system.

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References

1 Y. Takai, A. Kishimoto, M. Inoue and Y. Nishizuka, J. Biol. Chem. 252 (1977) 7603–7609.
2 Y. Takai, A. Kishimoto, U. Kikkawa, T. Mori and Y. Nishizuka, Biochem. Biophys. Res. Commun. 91 (1979) 1218–1224.
3 Y. Takai, A. Kishimoto, Y. Iwasa, Y. Kawahara, T. Mori and Y. Nishizuka, J. Biol. Chem. 254 (1979) 3692–3695.
4 Y. Nishizuka, Science 233 (1986) 305–312.
5 Y. Nishizuka, Nature 334 (1988) 661–665.
6 Y. Nishizuka, Science 258 (1992) 607–613.
7 M. Castagna, Y. Takai, K. Kaibuchi, K. Sano, U. Kikkawa and Y. Nishizuka, J. Biol. Chem. 257 (1982) 7847–7851.
8 C.L. Ashendel, Biochim. Biophys. Acta 822 (1985) 219–242.
9 P.J. Parker, L. Coussens, N. Totty, L. Rhee, S. Young, E. Chen, S. Stabel, M.D. Waterfield and A. Ullrich, Science 233 (1986) 853–858.
10 Y. Ono, T. Fujji, K. Ogita, U. Kikkawa, K. Igarashi and Y. Nishizuka, FEBS Lett. 226 (1987) 125–128.
11 Y. Ono, T. Fujji, K. Ogita, U. Kikkawa, K. Igarashi and Y. Nishizuka, J. Biol. Chem. 263 (1988) 6927–6932.
12 D. Schaap, J. Hsuan, N. Totty and P.J. Parker, Eur. J. Biochem. 191 (1990) 431–435.
13 S. Osada, K. Mizuno, T.C. Saido, Y. Akita, K. Suzuki, T. Kuroki and S. Ohno, J. Biol. Chem. 265 (1990) 22434–22440.
14 K.P. Huang, H. Nakabayashi and F.L. Huang, Proc. Natl. Acad. Sci. USA 83 (1986) 8535–8539.
15 M.D. Bazzi and G.L. Nelsestuen, Biochemistry 29 (1990) 7624–7630.
16 M.J. Berridge, Biochem. J. 220 (1984) 345–360.
17 M.J. Berridge, Annu. Rev. Biochem. 56 (1987) 159–193.
18 S.L. Pelech and D.E. Vance, Tr. Biochem. Sci. 14 (1989) 28–37.
19 J.H. Exton, J. Biol. Chem. 265 (1990) 1–4.

20 R. Zidovetzki and D.S. Lester, Biochim. Biophys. Acta 1134 (1992) 261–272.
21 Y.A. Hannun, C.R. Loomis and R.M. Bell, J. Biol. Chem. 260 (1985) 10039–10043.
22 B.R. Ganong, C.R. Loomis, Y.A. Hannun and R.M. Bell, Proc. Natl. Acad. Sci. USA 83 (1986) 1184–1188.
23 A. Azzi, D. Boscoboinik and C. Hensey, Eur. J. Biochem. 208 (1992).
24 M.D. Bazzi and G.L. Nelsestuen, Biochemistry 26 (1987) 1974–1982.
25 M.D. Bazzi and G.L. Nelsestuen, Biochemistry 30 (1991) 7970–7977.
26 J.M. Rodrigues-Paris M. Shoji, S. Yeola, D. Liotta, W.R. Vogler and J.F. Kuo, Biochem. Biophys. Res. Commun. 159 (1989) 495–600.
27 V. Brumfeld and D.S. Lester, Arch. Biochim. Biophys. 277 (1990) 318–323.
28 M.D. Bazzi and G.L. Nelsestuen, Biochemistry 26 (1987) 115–122.
29 D.S. Lester and V. Brumfeld, Int. J. Biol. Chem. 12 (1990) 251–256.
30 D.S. Lester and V. Brumfeld, Biophys. Chem. 39 (1991) 215–224.
31 E.H.W. Pap, P.I.H. Bastiaens, J.W. Borst, P. van den Berg, A. van Hoek, G.T. Snoek, K.W.A. Wirtz and A.J.W.G. Visser, Biochemistry (1993) in press.
32 G.T. Snoek, A. Feijen, W.J. Hage, W. Rotterdam and S.W. de Laat, Biochem. J. 255 (1988) 629–637.
33 K. Vos, A. van Hoek and A.J.W.G. Visser, Eur. J. Biochem. 165 (1987) 55–63.
34 F. Merola, R. Rigler, A. Holmgren and J.C. Brochon, Biochemistry, 28 (1989) 3383–3398.
35 A.K. Livesey and J.C. Brochon, Biophys. J. 52 (1987) 693–706.
36 N. Draper and H. Smith, Applied regression analysis (Wiley, New York, 1980).
37 Wolfram Research Inc., Mathematica 2.0 (Wolfram Research Inc., Champaign, IL, 1991).
38 D.W. Marquardt, Soc. Ind. Appl. Math. J. 11 (1963) 431–441.
39 R.E. Dale, J. Eisinger and W.E. Blumberg, Biophys. J. 26 (1979) 161–194.
40 M.D. Bazzi and G.L. Nelsestuen, Biochemistry 27 (1988) 7589–7593.
41 M. Lee and R.M. Bell, Biochemistry 30 (1991) 1041–1049.