Quantitation of the Interaction of Protein Kinase C with Diacylglycerol and Phosphoinositides by Time-Resolved Detection of Resonance Energy Transfer†

E. H. W. Pap,‡ P. I. H. Bastiaens,‡ J. W. Borst,‡ P. A. W. van den Berg,‡ A. van Hoek,§ G. T. Snoek,∥ K. W. A. Wirtz,∥ and A. J. W. G. Visser*,‡

Departments of Biochemistry and Molecular Physics, Agricultural University, Dreijenlaan 3, 6703 HA Wageningen, The Netherlands, and Centre for Biomembranes and Lipid Enzymology (CBLE), Utrecht University, Padulaan 8, 3584 CH Utrecht, The Netherlands

Received July 1, 1993; Revised Manuscript Received September 20, 1993®

ABSTRACT: Quantitative studies of the binding of protein kinase C (PKC) to lipid cofactors were performed by monitoring resonance energy transfer with time-resolved fluorescence techniques. For that purpose, diacylglycerol (DG), phosphatidylinositol 4,5-biphosphate (PIP_2), phosphatidylinositol 4-phosphate (PIP), phosphatidylinositol (PI), phosphatidylcholine (PC), and phosphatidylserine (PS) were labeled with a pyrenyl decanoyl moiety at the *sn-2* position of the lipid glycerol. These labeled lipids proved excellent energy acceptors of light-excited tryptophan residues in PKC. The quenching efficiency of the tryptophan fluorescence was determined as function of lipid probe concentration in mixed micelles consisting of poly(oxyethylene)-9-lauryl ether, PS, and various mole fractions of probe lipid. The experimental conditions and method of data analysis allowed the estimation of binding constants of single or multiple pyrene lipids to PKC. The affinity of PKC for inositide lipids increases in the order PI < PIP < PIP_2. The affinity of PKC for PIP and PIP_2 is higher than that for DG. Determination of PKC activity in the presence of labeled lipids and PS showed that only PIP_2 and DG activate PKC. Double-labeling experiments suggest that PIP_2 and DG are not able to bind simultaneously to PKC, indicating a reciprocal binding relationship of both cofactors. The results support the notion that, besides DG, PIP_2 can be a primary activator of PKC.

Protein kinase C (PKC)[1] has emerged as a crucial factor in transmembrane signal transduction influencing a great number of cellular processes including secretion, cell growth, and differentiation (Nishizuka, 1986; Kikkawa & Nishizuka, 1986). The interaction of PKC with specific lipids plays an essential role in the regulation of its activity. In vitro the enzyme is activated by PS in the presence of Ca^{2+} and DG. Activation is accompanied by binding of PKC to the membrane. This binding is controlled by strong electrostatic forces and weak hydrophobic interactions (Brumfeld & Lester, 1990). Although binding to the membrane occurs with any negatively charged phospholipid (König et al., 1985; Bazzi & Nelsestuen, 1987), PS seems to be the most effective in the binding reaction (Hannun et al., 1985; Ganong et al., 1986). In contrast to the binding reaction, the lipid requirement for the activation of PKC is highly specific, being met by DG and the tumor-promoting phorbol esters (Bazzi & Nelsestuen, 1988a, 1989; Brockerhoff, 1986). Recently, evidence has been obtained that besides these lipid cofactors PIP_2 is also able to activate PKC efficiently (O'Brain et al., 1987; Lee & Bell, 1991; Chauhan & Brockerhoff, 1988; Huang & Huang, 1991). At low PS concentrations the affinity of PKC for PIP_2 is higher than for DG while the maximal PKC activation is lower with PIP_2 than with DG (Lee & Bell, 1991; Chauhan & Brockerhoff, 1988; Huang & Huang, 1991). Different affinities toward PIP_2 were reported for the three major Ca^{2+}/PS/DG-dependent PKC species (Huang & Huang, 1991). In addition, it was found that both DG and PIP_2 inhibited the binding of phorbol-12,13-dibutyrate to PKC, which is indicative for a common binding site for PIP_2 and DG (Chauhan et al., 1989). In contrast, others observed that PIP_2 interacts at a site different from that of phorbol ester and concluded that the mechanism by which PIP_2 activates PKC is different from that of DG (Lee & Bell, 1991; Huang & Huang, 1991). The fact that PIP_2 may act as an activator of PKC as well has far reaching consequences for the regulation of PKC activity. In order to verify these observations, additional experiments are required to directly measure the binding of DG and PIP_2 to PKC, thereby allowing the determination of the binding constant of either lipid cofactor to PKC and providing the answer to the question of whether PIP_2 and DG bind to either one or two sites on the kinase. To achieve this goal, specific pyrene-labeled analogues of PIP_2 and DG were synthesized for use in studies in which the interaction with PKC was determined on line from time-resolved fluorescence detection of resonance energy transfer (RET). A major advantage of this technique is the direct and specific detection of interaction between PKC and the labeled cofactors in combination with high sensitivity and large dynamical range which allow the use of low concentrations of protein and lipid probes in the binding experiments. Verification of competition of pyrDG

† This research was supported by the Netherlands Foundation for Biophysics under the auspices of the Netherlands Organisation for Scientific Research (NWO) and by the Dutch Cancer Society (KWF).

* Address correspondence to this author at Department of Biochemistry, Agricultural University, Dreijenlaan 3, NL-6703 HA Wageningen, The Netherlands.

‡ Department of Biochemistry.

§ Department of Molecular Physics.

∥ Centre for Biomembranes and Lipid Enzymology.

® Abstract published in *Advance ACS Abstracts,* November 1, 1993.

[1] Abbreviations: ADC, analogue to digital converter; DOPC, dioleoylphosphatidylcholine; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; FWHM, full width at half-maximum; HEPES, *N*-(2-hydroxyethyl)-piperaxine-*N′*-2-ethanesulfonic acid; MEM, maximum entropy method; PC, phosphatidylcholine; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-phosphate; PIP_2, phosphatidylinositol 4,5-biphosphate; PKC, protein kinase C; PLC, phospholipase C; PS, phosphatidylserine; PTF, *p*-terphenyl; Pyr, pyrene decanoyl; RET, resonance energy transfer; TAC, time-to-amplitude converter; TLC, thin-layer chromatography; thesit, poly(oxyethylene)-9-lauryl ether; Tris, tris(hydroxymethyl)aminomethane.

0006-2960/93/0432-13310$04.00/0

and unlabeled DG for binding to PKC was performed in a parallel study, the results of which suggested that both molecules bind the same site (Bastiaens et al., 1993). The pyrene-labeled lipids were presented to PKC as part of a micellar system. This approach for investigating PKC–lipid interactions is well characterized (Hannun et al., 1985) and particularly suitable to determine the specificity and stoichiometry of the lipid requirement. The high concentration of micelles used validates the assumption that maximally one PKC molecule binds per micelle. Additional advantages of the use of micelles are that all (pyrene) lipids are exposed to the protein and that any effects that the physical state of a bilayer membrane may have on the interaction with the protein are eliminated.

EXPERIMENTAL PROCEDURES

Materials. Bovine brain L-α-phosphatidylserine (PS), dioleoyl-PC, diacylglycerol (DG), thesit [poly(oxyethylene)-9-lauryl ether], EGTA, HEPES, histone type IIIS, neomycin sulfate, phospholipase D from *Streptomyces species*, phospholipase C from *Bacillus cereus*, phenylmethanesulfonyl fluoride, Sephacryl S200, poly(lysine)-agarose, histone H1 from calf thymus, and trypsin from bovine pancreas (L-1-(tosylamino)-2-phenylethylchloromethylketone treated, 1.22 $\times 10^4$ BAEE units mg^{-1}) were supplied by Sigma Chemical Co. (St. Louis MO). Phenyl-Sepharose CL-4B and DEAE-Sepharose FF S200 were from Pharmacia (Uppsala, Sweden) and [γ-^{32}P]ATP (3000 Ci $mmol^{-1}$) was from Amersham (U.K.). The scintillation counting cocktail was from Packard (Meriden). All other phospholipids used were synthesized as described below. All solvents were distilled before use. All other chemicals were of reagent grade. Unless otherwise noted, experiments were performed using 20 mM Tris, pH 7.5, 50 mM KCl, and 20 μM EGTA at 20 °C.

Purification of PKC. Protein kinase C was purified from cytosolic extract of homogenized Wistar rat brains by a procedure similar to that described by Huang et al. (1986) and consecutively by DEAE, phenyl-Sepharose, Sephacryl S200, and poly(lysine)-agarose chromatography. The final preparation [with isozyme composition as described elsewhere (Huang et al., 1986; Sekiguchi et al., 1988)] was essentially pure, as demonstrated by silver staining of a polyacrylamide gel, and was stored at −70 °C in buffer (20 mM Tris, pH 7.9, 0.5 mM EGTA, 0.5 mM EDTA, 1 mM BME) with 25% glycerol (Merck, Darmstadt, Germany, fluorescence microscopy grade).

Assay of PKC Activity. The dependence of the Ca^{2+} and phospholipid-dependent PKC activity was assayed at a temperature of 20 °C, essentially as described by Snoek et al. (1988) using mixed micelles of 300 μM thesit, 5 mol % PS, and 0.01–4 mol % pyrene-labeled lipid.

Synthesis of Pyrene-Labeled Phosphoinositides. sn-2-(Pyrenyldecanoyl)-PI (pyrPI) was synthesized from yeast PI according to Somerharju et al. (1982, 1985). Pyrene-labeled analogues of phosphatidylinositol 4-phosphate (pyrPIP) and phosphatidylinositol 4,5-phosphate (pyrPIP$_2$) were synthesized from pyrPI using partially purified PI and PIP kinase preparations as described by Gadella et al. (1990). The products were identified as pyrPIP and pyrPIP$_2$ on TLC and from their elution behavior on immobilized neomycin columns. For structures of the compounds, see Figure 1.

Synthesis of Pyrene-Labeled Phosphatidylcholine, Phosphatidylserine, and Diacylglycerol. sn-2-(Pyrenyldecanoyl)-PC (pyrPC) was synthesized from egg-PC as described by Somerharju et al. (1982). Pyrene-labeled PS (pyrPS) was obtained enzymatically from pyrPC by transphosphatidylation

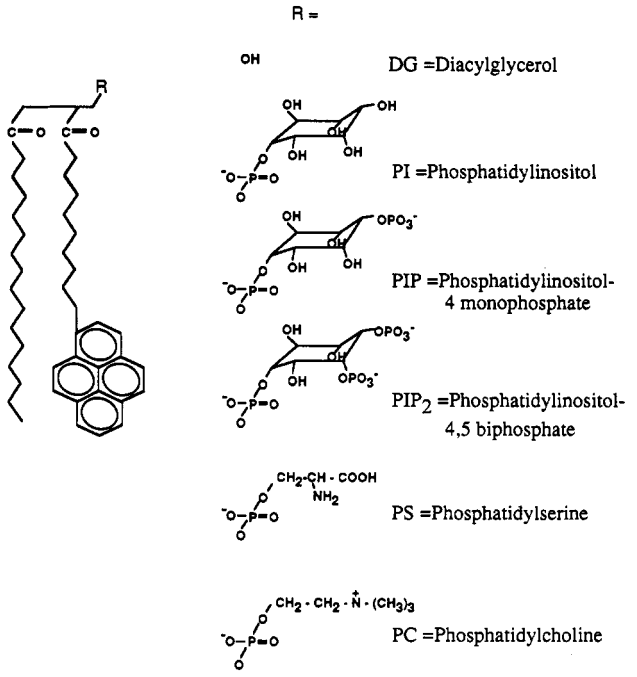

FIGURE 1: Chemical structures of the pyrene-labeled phospholipids.

catalyzed by phospholipase D (Comfurius et al., 1990). PyrDG was synthesized by a phospholipase C catalyzed hydrolysis of the diglyceride–phosphate linkage in pyrPC essentially as described by Myher and Kuksis (1984). Purification of the lipids was performed with high-performance liquid chromatography on a silicic acid column (240 × 10 mm, LiChroprep Si 60, Merck, Germany). Elution was performed with an increasing methanol gradient in chloroform (0–40%). Structures of the compounds used are given in Figure 1.

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 solubilization in buffer [1 mM thesit, 20 mM Tris-HCl (pH 7.4), 50 mM KCl, and 20 μM EGTA] by vortexing and brief bath sonication. In the binding studies the thesit concentration was 100 μM and the PS concentration was 5 μM (5 mol %). The fluorescent lipid concentration ranged from 0.01 to 4 μM (0.01–4 mol %). The phospholipid content was determined by phosphate analysis according to the method of Rousser et al. (1970). The pyrene concentration was estimated by measuring the absorption at 342 nm in ethanol/DMSO (75:25 v/v) (ϵ = 39700 M^{-1} cm^{-1}). In the fluorescence experiments the PKC concentration was 80 nM.

Instrumental. Time-resolved fluorescence measurements were carried out using a time-correlated single photon counting set up as described earlier (Bastiaens et al., 1992). A mode-locked cw YLF laser (Coherent model Antares 76-YLF, Palo Alto, CA) that was equipped with an LBO frequency doubler to obtain output at 527-nm wavelength was used for the synchronously pumping of a cavity-dumped Rhodamine 6G dye laser (Coherent model 701-2 CD). The light output of the dye laser was frequency doubled using a BBO crystal (3 × 4 × 7 mm^3, Gsänger, Planegg, Germany). A variable waveplate (New Focus model 5540, Mountain View, CA) was used to rotate the polarization of the vertically polarized output light of the dye laser to horizontally polarized light to have again vertically polarized (UV) light available for excitation after the type I frequency doubling in the BBO crystal. The repetition rate of excitation pulses was 951 kHz, the excitation wavelength 295 nm, the duration about 4 ps

FWHM, and the pulse energy in the tens of pJ range. Fluorescence was selected using a 3-mm WG 335 cut-off filter (Schott, Mainz, Germany), an interference filter at 348.8 nm (Schott, bandwidth 4.8 nm FWHM), and a sheet type polarizer (Polaroid type HNP'B, Cambridge, MA) that could be rotated under computer control to parallel and perpendicular with respect to the direction of excitation polarization. Single-photon responses from the microchannel-plate photomultiplier (Hamamatsu, model 1645U, Hamamatsu, Japan) were amplified by a wide band amplifier (Hewlett Packard model 8447F, Palo Alto, CA), analzyed in one channel of a quad constant fraction discriminator (Tennelec modified model TC 454, Oak Ridge, TN) and then used as a start signal for the time-to-amplitude converter (TAC, Tennelec model TC 864). The stop signal for the TAC was generated using another channel of the TC 454, driven by the pulses from a fast PIN photodiode (Hewlett Packard model 5082-4203 at 45 V reverse bias) that was excited with red light left from the frequency doubling set up (van Hoek & Visser, 1990). The output signal of the TAC was analyzed by an analogue-to-digital converter (Nuclear Data model ND 582, a 1.5-μs fixed death time ADC, Schaumburg, IL), and the output signal of the ADC was gathered in a multichannel analyzer (Nuclear Data model ND66); 1024 channels were used per experimental decay with a time spacing of 30 ps per channel. Measurements consisted of a number of sequences of measuring during 10-s parallel and 10-s perpendicular polarized emission. After the fluroescence of a sample was measured, the background emission of PKC-free samples was measured, at one-fifth of the time of sample acquisition, and then used for background subtraction. PTF (BDH, Poole, U.K.) dissolved in ethanol, 1.06-ns single-exponential decay time (Vos et al., 1987), served as a reference compound to yield the dynamic instrumental response function of the set up (van Hoek & Visser, 1985). The sample temperature was 20 °C.

Computational Methods. The inverse Laplace transform of the fluorescence decay [$I(t)$] of the enzyme [the spectrum of contributions $\alpha(\tau)$ to the lifetimes (τ)] was obtained with the commercially available maximum entropy method (Maximum Entropy Data Consultants Ltd., Cambridge, U.K.):

$$I(t) = \int_0^\infty \alpha(\tau) e^{-t/\tau}\, d\tau \tag{1}$$

The principle of this method is fully described in previous publications (Gentin et al., 1990; Livesey et al., 1987; Brochon et al., 1992; Bastiaens et al., 1992).

Since PKC contains multiple tryptophan residues [eight for PKC from bovine brain (Parker et al., 1986)], the fluorescence decay is complex, and no unique lifetime class arises in the lifetime distribution of PKC without or with quenching by RET. Upon quenching by RET, however, several lifetime peaks are shifted to shorter time, which makes it difficult to determine the fraction of lipid sites occupied with pyrene lipids from analysis of the lifetime spectra. Therefore the average fluorescence lifetime $\langle\tau\rangle$ was used as an observable to monitor the occupancy of a PKC molecule with pyrene lipids. The average lifetime was calculated from the lifetime spectra according to

$$\langle\tau\rangle = \frac{\sum_{i=1}^{s} \alpha_i \tau_i}{\sum_{i=1}^{s} \alpha_i} \tag{2}$$

where the summation is carried out over the whole range (s) of τ_i values of an $\alpha(\tau)$ spectrum. The fluorescence quenching of tryptophan residues of PKC by pyrene lipids is described as follows. Vacant lipid sites at the protein surface and pyrene lipids in the micelle are in binding equilibrium. The fluorescence lifetimes are assumed to be short relative to the mean residence time of a pyrene lipid in a lipid "site" on the protein, and it is assumed that only pyrene lipid molecules that interact with PKC contribute to the tryptophan quenching. This is a reasonable assumption, since the characteristic critical Förster distance for a pyrene–tryptophan couple was calculated to be 2.7 nm as compared with the dimensons of a spherical PKC molecule with a hydrodynamic diameter of $\approx$6 nm, and of a micelle with a diameter of $\approx$7 nm (both dimensions are based on measured rotational correlation times, data not shown). The average distance between pyrene lipids randomly distributed within the micelle and the protein is thus too large for efficient energy transfer. Furthermore, it is assumed that binding of pyrene lipid to a "site" on the protein contributes additively to the total fluorescence quenching rate, resulting in a shortening of the average fluorescence lifetime. A binding equation can be deduced in which γ is defined as the fraction of sites occupied by a pyrene lipid. Since the concentration of PKC is an order of magnitude lower than the concentration of micelles, the probability that more than one PKC molecule binds to one micelle can be neglected, resulting in a binding equation independent of the PKC concentration. Similarly, the probability that more than one pyrene lipid is present in a micelle can be neglected at low concentrations of pyrene lipid. When we assume reversible lipid binding to a single site at the PKC molecule, the lipid binding to this site is simply governed by first-order dissociation (k^-) and association rate constants (k^+), and the equilibrium constant K for the binding becomes

$$K = \frac{k^-}{k^+} = \frac{(1-\gamma)(1-\gamma)}{\gamma} \tag{3}$$

When each PKC molecule has m equivalent and independent lipid binding sites, and n pyrene lipids are allowed in the micelle, the units of the association rate constant become second order, and the dissociation rate constant remains first order. The average number of free sites per micelle occupied with a PKC molecule can then be expressed as $m(1-\gamma)$, and the average number of free lipid molecules per micelle equals $(n - m\gamma)$, where $(n \geq m\gamma)$. The equilibrium constant K becomes

$$K = \frac{(1-\gamma)(n-m\gamma)}{\gamma} \tag{4}$$

Solving the quadratic equation for $\gamma(n,m)$, one obtains

$$\gamma(n,m) = \frac{1}{2}\left[\left(1 + \frac{K}{m} + \frac{n}{m}\right) - \sqrt{\left(\frac{-4n}{m}\right) + \left(-1 - \frac{K}{m} - \frac{n}{m}\right)^2}\right] \tag{5}$$

When the rate of dissociation of the complex is larger than the rate of exchange of pyrene lipids between micelles, the lipid molecules have to be considered as compartmentalized reactants, in which the probability (P_n) of finding n lipid cofactor molecules in a micelle of size S (surfactant number) at a labeling ratio (χ_T) is binomially distributed:

$$P_n = \frac{S!}{n!(S-n)!} \chi_T^{\,n} (1-\chi_T)^{S-n} \tag{6}$$

The total fraction of occupied lipid sites at a given labeling

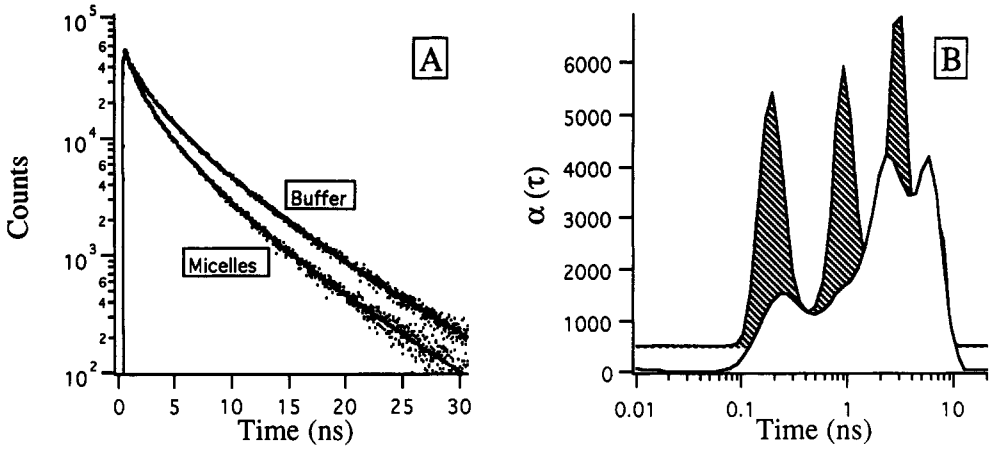

FIGURE 2: (A) Experimental and fitted decays of tryptophan fluorescence of PKC in buffer (80 nM) and in the presence of 100 μM thesit micelles containing 5 mol % PS and 4 mol % pyrPIP$_2$. (B) The MEM-recovered lifetime distributions of tryptophan fluorescence of PKC in buffer ("front", open peaks) and in the presence of the above-mentioned mixed micelles ("rear", hashed peaks). The measurements were performed in 20 mM Tris buffer (pH 7.5, 50 mM KCl and 1 mM $CaCl_2$) at 293 K.

ratio and number of lipid sites per PKC molecule is then given by

$$\gamma_{tot} = \sum_{n=1}^{S} P_n \gamma(n,m) \quad (7)$$

When this model was applied to the experimental data, the equilibrium constant K was determined from the dependence of the average lifetime on the mole fraction of pyrene-labeled PIP, PIP$_2$, and DG in terms of three adjustable parameters b, K, and X for m = 1–6:

$$\langle \tau \rangle = b - X\gamma_{tot} \quad (8)$$

where fitting parameter b corresponds to the average lifetime of the nonquenched PKC and X to the decrease in average lifetime when PKC is maximally quenched.

RESULTS

Tryptophanyl Fluorescence Decay and Distribution of Lifetimes. Figure 2 shows the experimental fluorescence decay and the analysis in a distribution of lifetimes of PKC in the presence and absence of pyrPIP$_2$ in thesit–PS mixed micelles. In all experiments the residuals of the fits were randomly scattered around zero, indicating an optimal fit (results not shown). As expected, the fluorescence decay of tryptophan in this enzyme is complex (note that the vertical scale in Figure 2 is logarithmic), probably because there are multiple classes of emitting tryptophan residues in the protein. In all analyses at least three peaks appeared in the lifetime distribution which may originate from three subclasses of tryptophan emitters. Addition of 4 mol % pyrPIP$_2$ to mixed micelles containing 5 mol % PS caused quenching of tryptophan fluorescence as manifested by a more rapid fluorescence decay. A substantial reduction of the barycenter values for each lifetime class is observed, indicating that each subclass of emitters is within the range of critical energy transfer distance of the pyrene moiety of PIP$_2$. The average lifetime of tryptophan fluorescence in the enzyme was found to be independent of the concentration of mixed micelles above 100 μM thesit with 5 mol % PS and 1 mol % pyrDG (Figure 3), revealing that saturation of binding is approached at this lipid concentration. The presence of 5 mol % brain PS in the micelles apparently provides sufficient binding capacity for PKC. This was confirmed in an identical system using fluorescence correlation spectroscopy (Bastiaens et al., 1993). Thus we further assume that PKC is associated with the micelles if Ca^{2+} and 5 mol % PS are present, independent of the pyrene–lipid concentration.

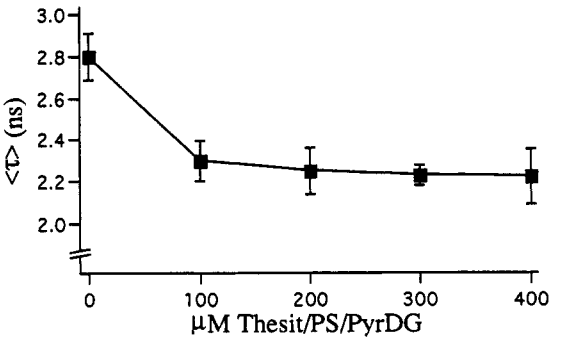

FIGURE 3: Average fluorescence lifetime ($\langle \tau \rangle$) of PKC as a function of the concentration thesit with 5 mol % PS and 1 mol % pyrDG. The experiment was performed in 20 mM Tris buffer (pH 7.5, 50 mM KCl, and 1 mM $CaCl_2$) at 293 K.

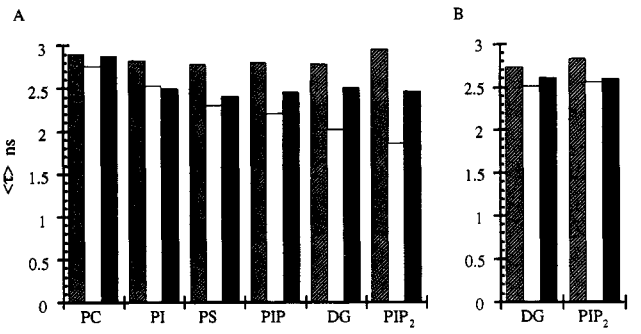

FIGURE 4: Comparative effects of various pyrene-labeled phospholipids in micelles on the average fluorescence lifetime of tryptophan residues in PKC. PKC was incubated with 100 μM mixed micelles containing 1 mol % pyrene lipid in the presence of 5 mol % PS (A) and in the presence of 5 mol % DOPC (B). The measurements were performed by adding successively 10 μM EGTA (hashed bars), 0.5 mM Ca^{2+} (□) and 10 mM EGTA (■).

Ca^{2+} and PS Dependence of PIP$_2$ and DG Binding to PKC. In order to examine the relationship between Ca^{2+} requirement and pyrene lipid binding, the average lifetime of tryptophan fluorescence in PKC was measured sequentially in the absence of Ca^{2+}, in the presence of Ca^{2+}, and then in the presence of saturating amounts of EGTA. In the absence of Ca^{2+} (20 μM EGTA) no effect of pyrene lipids is observed (Figure 4A). The average lifetimes of tryptophan fluorescence, obtained in the presence of the various pyrene lipids, display values scattered around the one obtained with micelles not loaded with probe lipid. Addition of Ca^{2+} caused only a slight

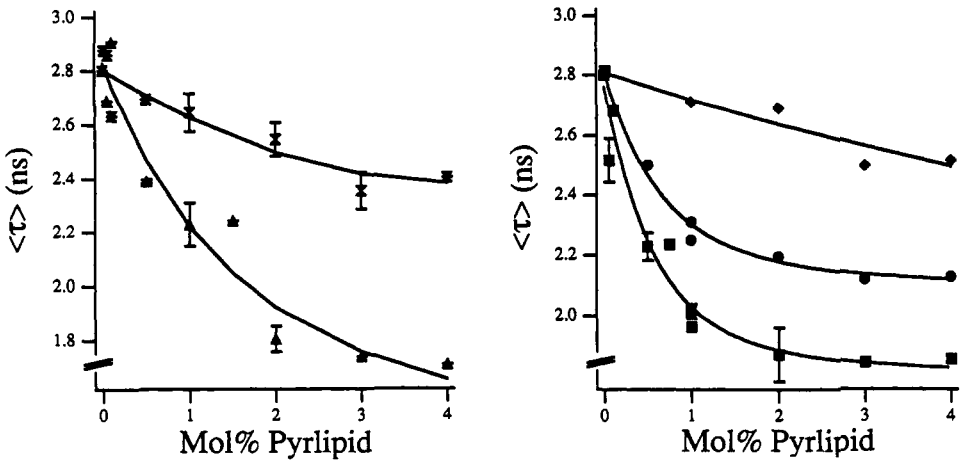

FIGURE 5: Dependence of the average fluorescence lifetime of tryptophan residues in PKC on the mole fraction of pyrene-labeled PIP_2 (■), PIP (●), PI (◆), PS (↕), and DG (▲) in 100 μM mixed micelles containing 5 mol % PS. The measurements were performed in 20 mM Tris buffer (pH 7.5, 50 mM KCl and 1 mM $CaCl_2$) at 293 K.

decrease of the average lifetime of tryptophan fluorescence when micelles with 2 mol % of pyrPC of pyrPI were present. In case of micelles containing 2 mol % of pyrene-labeled PS, PIP, DG, and PIP_2, a substantial reduction of the fluorescence lifetime is observed upon addition of Ca^{2+}. The effect of Ca^{2+} on the quenching of tryptophan fluorescence by the pyrene lipids decreases in the order PIP_2 > DG > PIP > PS. As is shown in the last column of Figure 4A, this effect can be largely reversed by removal of Ca^{2+} with EGTA. The effect of Ca^{2+} on the activation of PKC by the above-mentioned lipids revealed a qualitatively similar result (see below). On removal of Ca^{2+} by EGTA, the average lifetime increases to almost 90% of the value obtained before addition of Ca^{2+}, demonstrating major reversibility of PKC–lipid cofactor binding as was also found by other groups (Bazzi & Nelsestuen, 1987, 1991). In addition, the binding of pyrDG and $pyrPIP_2$ was investigated in the absence of 5 mol % brain PS. As can be seen in Figure 4B, PS is an absolute prerequisite for the interaction of the protein with its lipid cofactors DG and PIP_2. The simplest explanation of these results is that, for activation by pyrDG and $pyrPIP_2$, direct interaction of these cofactors with PKC is required. This interaction is not established in the absence of either PS or Ca^{2+} [see also Bazzi and Nelsestuen (1987) and Rodriguesz-Paris et al. (1989)]. Conversely, mixed micelles containing 5 mol % PS are fully capable of binding PKC in the presence of Ca^{2+}. Clearly, Ca^{2+} is needed for the interaction of PKC with PS. Binding of PKC to PS molecules precedes the interaction with pyrene lipid cofactors, which then leads to activation of PKC.

Fluorescence Quenching by the Various Pyrene Lipids. The dependence of tryptophan fluorescence quenching of PKC on the mole fraction of pyrene–lipids were examined in a range of 0–4 mol %. The data were analyzed in terms of lifetime distributions, and the average fluorescence lifetime $\langle\tau\rangle$ was calculated using eq 2. In Figure 5 the average lifetime is plotted as a function of the pyrene–lipid mole fraciton for the various lipid analogues. A steep decrease in the plot of the average lifetime against the mole fraction of pyrene–lipid indicates a high affinity between PKC and the lipid, while the average lifetime at saturating levels of pyrene–lipids is governed by the eventual binding configuration and stoichiometry. The quenching characteristics of pypPIP, $pyrPIP_2$, and pyrDG are different from those of pyrPI and pyrPS in two aspects. First, within the mole fraction range saturation of the quenching effect is approached for pyrPIP, $pyrPIP_2$, and pyrDG in strong contrast with the almost linear dependence of the average lifetime on the mole fraction of pyrene-labeled PI and PS. This difference implies that the affinity of PKC

Table I: Values of the Equilibrium Constant K for the Binding of Single (m = 1) or Multiple (m = 2–6) Pyrene-Labeled PIP_2, PIP, and DG Molecules to PKC and the Quality of the Fit (χ^2), Obtained from the Analysis of the Average Fluorescence Lifetime Dependence on the Mole Fraction of These Pyrene Lipids

	PIP_2		PIP		DG	
m	K	χ^2	K	χ^2	K	χ^2
1	0.431	0.0053	0.676	0.0016	3.2	0.0146
2	0.119	0.0053	0.264	0.0013	2.58	0.0145
3	2×10^{-4}	0.0059	0.003	0.0013	1.84	0.0144
4	6×10^{-4}	0.0090	6×10^{-4}	0.0037	1.01	0.0143
5	8×10^{-5}	0.0139	8×10^{-5}	0.0086	0.43	0.0141
6	2×10^{-5}	0.0197	4×10^{-5}	0.0142	0.052	0.0141

for pyrPI and pyrPS is considerably lower than for pyrPIP, $pyrPIP_2$, and pyrDG. Second, the average lifetimes obtained at higher concentrations of $pyrPIP_2$ and pyrDG are substantially shorter as compared to the other lipids. The lifetime value at saturating amounts of pyrPIP seems to be intermediate between those of pyrPI and $pyrPIP_2$. The quenching of PKC fluorescence by pyrPIP, $pyrPIP_2$, and pyrDG was suitable for the determination of the binding constant because quenching approached saturation with these lipids within the experimental mole fraction range. From the dependence of the average lifetime on the mole fraction of pyrene lipid in the micelles, the binding constants were determined for 1–6 lipid binding sites (m) per PKC molecule, according to the model as described under Experimental Procedures (eq 8). In this analysis it was assumed that a micelle consists of 300 molecules (S = 300), on the basis of fluorescence correlation spectroscopic studies with an identical micellar system (Bastiaens et al., 1993). The results of the fit are listed in Table I. The quality of the analysis of the average lifetime dependence on the mole fraction of pyrene-labeled PIP and PIP_2 decreases considerably when more than three binding sites were assumed per PKC molecule. From the standard error of the average lifetime values and the number of degrees of freedom, the significant increase of χ^2 from its minimum value can be determined at a confidence interval of 0.67 according to a so-called fstat test (Beechem et al., 1991). With this approach, χ^2 limits of 6.8×10^{-3}, 2.2×10^{-3}, and 1.9×10^{-2} were obtained for $pyrPIP_2$, pyrPIP, and pyrDG, respectively. It can thus be concluded that the pyrPIP and $pyrPIP_2$ data only allow adequate fits if three or less binding sites per PKC molecules are assumed. Since the lifetime dependency on the mole fraction of pyrDG is less pronounced than for the former two lipids, the number of DG sites has no clear effect on the quality of the fit. Apparently, the quality of the data does not allow determi-

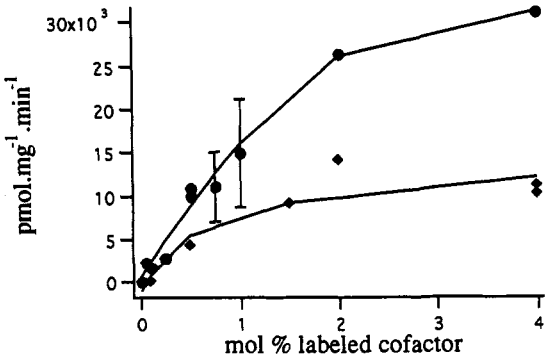

FIGURE 6: PIP_2- (●) or DG- (◆) stimulated PKC activity in the presence of labeled cofactors. The activity is expressed in nmol of phosphate incorporated into histone IIS/(min·mg of PKC).

nation of the absolute number of DG binding sites. For each value of m, however, the equilibrium constant of PKC for pyrPIP and pyrPIP_2 is considerably lower than that for pyrDG, indicating a higher affinity for either phosphoinositide than for DG.

Activation of PKC by Pyrene-Labeled Lipids. The dependency of PKC activation of pyrene-labeled lipids in the presence of 0.5 mM Ca^{2+} and 5 mol % brain PS was measured in the range of 0–4 mol %, in parallel with the fluorimetric binding studies. Without Ca^{2+} and 5 mol % PS, no significant PKC activity was found. The activity profiles with either effector, pyrDG or pyrPIP_2, in the presence of 5 mol % PS were found to be quite different. The maximal activity attained with pyrPIP_2 is higher than that achieved by pyrDG (Figure 6). None of the other pyrene-labeled phospholipids supported activation even up to 4 mol %. Therefore the activation of PKC displays, in agreement with the binding studies, high specificity for pyrDG and pyrPIP_2. In addition, these results refute the possibility of pyrene itself being the activating factor in the fluorescent lipids. The activity of the enzyme is roughly proportional to the fraction of PKC bound to pyrPIP_2 and pyrDG (Figure 6).

Double-Labeling Experiments. In order to investigate the relationship between DG and PIP_2 binding to PKC, double-labeling experiments were performed by monitoring the tryptophan fluorescence decays in the presence of various molar ratios of pyrDG and pyrPIP_2 while keeping the total concentration of pyrene cofactor constant. In this approach it is assumed that each pyrene lipid that binds to PKC quenches the protein fluorescence additively. This approach was preferred over competition experiments using nonlabeled lipid cofactors since possible effects of the pyrene moiety on the binding to PKC are then omitted. If both cofactors interact independently with PKC at separated sites, a more efficient quenching is expected at equal molar ratios than when one of the cofactors is solely present. However, when the lipid cofactors interfere in each other's binding, e.g., when they share the same binding "site", no effect is expected from a change in the molar ratios of the two pyrene cofactors. In these double-labeling experiments, the total amount of lipid cofactors must be high in order to eliminate micelles containing only one of the cofactors. Figure 7 shows the tryptophan average fluorescence lifetime as a function of the molar ratio of pyrDG and pyrPIP_2 at 3.5 and 5 mol % total pyrene lipid cofactor. The tryptophan average lifetimes are scattered around a value of 1.8 and 1.7 ns, respectively. The dependency of the lifetime values on the molar ratios is found to be linear, rather than "parabolic", indicating that the binding of either cofactor inhibits the binding of the other one.

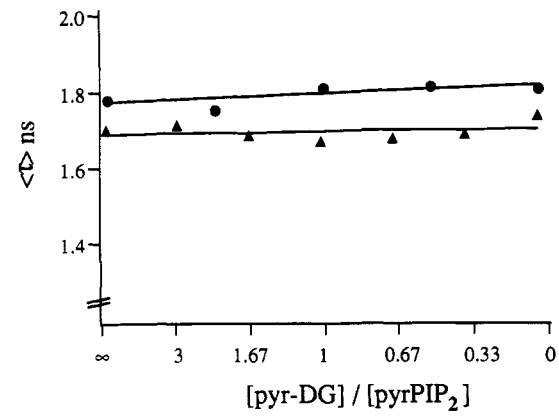

FIGURE 7: Double-binding experiments with pyrPIP_2 and pyrDG. Binding of lipid cofactors to PKC was measured in thesit mixed micelles containing PS at 5 mol % at various molar ratios of pyrDG to pyrPIP_2 while keeping the total concentration of pyrene-labeled cofactor constant at 3.5 (●) and 5 (▲) μM.

DISCUSSION

The binding of DG, PIP_2, and various other lipids to PKC was investigated by measuring the quenching of tryptophan fluorescence in PKC as a result of radiationless energy transfer to pyrene-labeled analogues of the natural lipids in mixed micelles. The results of the lipid titration experiments were quantified in terms of number of lipid binding sites per PKC molecule and binding constants for the lipid cofactors. In addition, the effect of the presence of both labeled cofactors at various relative concentrations on the average lifetime of PKC was studied in double-labeling experiments. Our results show that the affinity of PKC for lipids decreases in the order PIP_2 > PIP > DG > PI, PS, PC. The affinity of PKC for DG is relatively low (binding constant is 3.2 molecules, m = 1) as compared to its affinity to PIP and PIP_2. In a parallel study, however, using 10 mol % PS in the mixed micelles, a binding constant of 0.15 molecules (m = 1) for the interaction of PKC with pyrDG is reported (Bastiaens et al., 1993), indicating that binding of PKC to DG is highly dependent on the mole fraction PS in the micelles [see also Lee and Bell (1991)]. When the multiple sites are encompassed in our model, identical binding characteristics are assumed. This is justified by the hyperbolic dependence of the binding isotherms without inflection points. The exact number of lipid sites could not be determined from the pyrDG data, but in the case of pyrPIP_2 and pyrPIP this number is probably less than three, confirming the results of Huang and Huang (1991). Other workers reported that the lipid cofactor DG binds to PKC in a one to one stoichiometry (Brockerhoff et al., 1986; Gschwendt et al., 1991; Hannun et al., 1985; Ganong et al., 1986). Of all the lipid analogues examined, only PIP_2 and DG were able to activate PKC. Although the affinity of PKC for PIP is high, no activation of PKC is induced by the interaction with this lipid, indicating that strong binding is insufficient for activation and that special features of DG and PIP_2 lead to PKC activity during the interaction. This is supported by the observation that the tryptophan fluorescence of PKC in the presence of saturating amounts of pyrPIP is considerably less quenched than in the presence of saturating amounts of pyrPIP_2 or pyrDG, indicating a different binding configuration or stoichiometry. Since the analysis of the tryptophan average lifetime dependence on the mole fraction of PIP does not allow more than three PIP sites per PKC molecule, in agreement with PIP_2, both inositol lipids might share the same binding site(s) on PKC. The binding of PIP_2 to these site(s) might, like DG, stabilize an active PKC conformation in which the tryptophan residues are transferring energy more efficiently

to pyrene. The high-affinity binding of its precursor PIP to these sites, however, is not competent to stabilize this active and more "quenchable" protein conformation. These stabilizing features may arise from favorable geometrical structures, as reported for DG (Kerr et al., 1987; Ono et al., 1989), or from electrostatic interactions (in case of PIP_2) satisfying particular binding conditions. The effect of conformational changes of PKC induced by cofactor molecules on the equilibrium constant of binding has to be systematically investigated further. If binding of the cofactors is not reversible, the model cannot be applied.

Besides the structural requirements of the lipid cofactors, it is reported that PKC can be activated by membrane bilayer perturbations induced by DG [summarized by Zidovetzki and Lester (1992)]. In this respect it is worth noting that there is evidence that both PIP_2 and DG are both capable of forming nonbilayer lipid phases (Hendrickson, 1969; Das et al., 1986; De Broeck & Zidovetzki, 1989). Similar physicochemical disturbances of the bilayer by both cofactors could increase the accessibility of hydrocarbon regions of the membrane for PKC, thereby intensifying hydrophobic interactions, possibly accompanied by conformational changes. However, since in this study the lipid cofactors were incorporated in less ordered mixed micelles, where specific structural membrane arrangements or lipid phases are absent, the particular molecular features of DG and PIP_2 seem to be absolutely required and sufficient for PKC activation.

Contradictory conclusions were reported concerning the shared or separate site of PIP_2 and DG on PKC. These reports are based on observations that PIP_2 does (Chauhan et al., 1989) or does not (Lee & Bell, 1991; Huang & Huang, 1991) inhibit phorbol-12,13-dibutyrate binding. Although the reason for these different experimental results is unknown, the double-labeling experiments in this paper provides a direct approach to show the mutual dependence of the binding of both cofactors to PKC. Since no additive fluorescence quenching was found when both labeled cofactors were present at equal molar ratios, as compared with the quenching in the presence of one of the labeled cofactors, simultaneous binding of both cofactors to PKC does not seem to be probable. In the shared binding site model, competition in binding is in agreement with the double-labeling results. The occupation of a site by one of the cofactors simply prevents the binding of the other cofactor. However, the separate site model is not rigorously rejected by these results. If there are separate sites for DG and PIP_2, the binding of one of these lipids to the protein could stabilize the active PKC conformation which is not able to bind the other cofactor lipid. In addition, allosteric effects or steric effects of the cofactors at closely spaced sites may prevent the binding of the other lipid cofactor. Our results strongly support the notion that PIP_2 has a dual role in the activation mechanisms of PKC (Lee & Bell, 1991; Chauhan et al., 1989). First, PIP_2 can function as a precursor for DG in phospholipase C catalyzed hydrolysis. In this role PIP_2 itself is inert and only its hydrolysis is the switch for the PKC activation. In a later stage of the activation other lipids can also function as a precursor for DG (Nishizuka, 1992). Second, PIP_2 itself is a high-affinity ligand and effective activator of PKC in the presence of Ca^{2+}. This implies that PKC can be activated independently from the inositide breakdown route when Ca^{2+} is provided via alternative routes (like Ca^{2+} channels in the plasma membrane). Reports of oscillatory cytosolic Ca^{2+} waves, independently of stimulated inositol 1,4,5-trisphosphate formation (Rooney et al., 1991), are consistent with this second model of activation. In the DG-dependent activation, the time response of PKC activity depends mainly on the presence of DG (Bazzi & Nelsestuen, 1988a; Nishizuka, 1986, 1992), while in the PIP_2-dependent activation, regulation of PKC activity is probably controlled by the temporal availability of Ca^{2+} in the cytosol. Regulation of the kinase activation via the availability of PIP_2 by PIP-kinase and PLC, responsible for the synthesis and degradation of PIP_2, is not very likely, since the degradation of PIP_2 leads automatically to the generation of DG, which is at least an equally competent activator of the kinase. This would thus only be useful if the activation of PKC by PIP_2 and DG would lead to an active kinase with distinct characteristics, like substrate specificity. Physiological significance of the activation of PKC by PIP_2, however, will require additional cellular studies focused on PKC activation independent from the inositol breakdown route.

REFERENCES

Bastiaens, P. I. H., van Hoek, A., Benen, J. A. E., Brochon, J. C., & Visser, A. J. W. G. (1992) *Biophys. J. 63*, 839.

Bastiaens, P. I. H., Pap, E. H. W., Borst, J. W., van Hoek, A., Kulinski, T., Rigler, R., & Visser, A. J. W. G. (1993) *Biophys. Chem.* (in press).

Bazzi, M. D., & Nelsestuen, G. L. (1987) *Biochemistry 26*, 115.

Bazzi, M. D., & Nelsestuen, G. L. (1988a) *Biochemistry 27*, 7598.

Bazzi, M. D., & Nelsestuen, G. L. (1988b) *Biochem. Biophys. Res. Commun. 152*, 336.

Bazzi, M. D., & Nelsestuen, G. L. (1989) *Biochemistry 28*, 3577.

Bazzi, M. D., & Nelsestuen, G. L. (1991) *Biochemistry 30*, 7970.

Beechem, J. M., Gratton, E., Ameloot, M., Knutson, J. R., & Brand, L. (1991) in *Topics in Fluorescence Spectroscopy* (Lakowicz, J. R., Ed.) Vol. 2, p 241, Plenum Press, New York.

Brochon, J. C., Mérola, F., & Livesey, A. K. (1992) in *Synchrotron Radiation and Dynamic Phenomena*, p 435, American Institute of Physics, New York.

Brockerhoff, H. (1986) *FEBS Lett. 201*, 1.

Brumfeld, V., & Lester, D. S. (1990) *Arch. Biochem. Biophys. 227*, 318.

Chauhan, A., Chauhan, V. P. S., Deshmukh, D. S., & Brockerhoff, H. (1989) *Biochemistry 28*, 4952.

Chauhan, V. P. S., & Brockerhoff, H. (1988) *Biochem. Biophys. Res. Commun. 155*, 18.

Comfurius, P., Bevers, E. M., & Zwaal, R. F. A. (1990) *J. Lipid Res. 31*, 17–19.

Das, S., & Rand, R. P. (1986) *Biochemistry 25*, 2882.

De Boeck, H., & Zidovetzki, R. (1989) *Biochemistry 28*, 7439.

Gadella, T. W. J., Moritz, A., Westerman, J., & Wirtz, K. W. A. (1990) *Biochemistry 29*, 3389.

Ganong, B. R., Loomis, C. R., Hannun, Y. A. & Bell, R. M. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 1184.

Gentin, M., Vincent, M., Brochon, J. C., Livesey, A. K., Cittanova, N., Gallay, J. (1990) *Biochemistry 29*, 10405.

Hannun, Y. A., Loomis, C. R., & Bell, R. M. (1985) *J. Biol. Chem. 260*, 10039.

Hendrickson, H. E. (1969) *Ann. N.Y. Acad. Sci. 165*, 668.

Huang, F. L., & Huang, K. P. (1991) *J. Biol. Chem. 266*, 8727.

Huang, K. P., Nakabayashi, H., & Huang, F. L. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 8535.

Hubbard, S. R., Bishop, W. R., Kirschmeier, P., George, S. J., Cramer, S. P., & Hendrickson, W. A. (1991) *Science 254*, 1776.

Kerr, D. E., Kissinger, L. F., Gentry, L. E., Purchio, A. F., & Shoyab, M. (1987) *Biochem. Biophys. Res. Commun. 148*, 776.

Kikkawa, U., & Nishizuka, Y. (1986) *Annu. Rev. Cell Biol. 2*, 149.

König, B., Dinitto, P. A., & Blumberg, P. M. (1985) *J. Cell. Biochem. 27*, 165.

Lee, M. H., & Bell, R. M. (1991) *Biochemistry 30*, 1041.

Livesey, A. K., & Brochon, J. C. (1987) *Biophys. J. 52*, 693.

Myher, J. J., & Kuksis (1984) *J. Biochem. Cell Biol. 62*, 352.

Nishizuka, Y. (1986) *Science 233*, 305.

Nishizuka, Y. (1992) *Science 258*, 607.
O'Brain, C. A., Arthur, W. L., & Weinstein, I. B. (1987) *FEBS Lett. 214*, 339.
Ono, Y., Fujii, T., Igarashi, K., Kuno, T., Tanaka, C., Kikkawa, U., & Nishizuka, Y. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 4868.
Parker, P. J., Coussens, L., Totty, N., Rhee, L., Young, S., Chen, E., Stabel, S., Waterfield, M. D., & Ullrich, A. (1986) *Science 233*, 853.
Rodriguesz-Paris, J. M., Shoji, M., Yeola, S., Liotta, D., Vogler, W. R., & Kuo, J. F. (1989) *Biochem. Biophys. Res. Commun. 59*, 495.
Rooney, T. A., Renard, D. C., Sass, E. J., & Thomas, A. P. (1991) *J. Biol. Chem. 266*, 12272.
Rousser, G., Fleischer, S., & Yamamoto, A. (1970) *Lipids 5*, 494.
Sekiguchi, K., Tsukuda, M., Ase, K., Kikkawa, U., & Nishizuka, Y. (1988) *J. Biochem. (Tokyo) 103*, 759.
Snoek, G. T., Feijen, A., Hage, W. J., Rotterdam, W., & de Laat, S. W., (1988) *Biochem. J. 255*, 629.
Somerharju, P. J., & Wirtz, K. W. A. (1982) *Chem. Phys. Lipids 30*, 81.
Somerharju, P. J., Virtanen, J. A., Elkund, K., Viano, P., & Kunnunen, P. K. J. (1985) *Biochemistry 24*, 2773.
Vos, K., Van Hoek, A., & Visser, A. J. W. G. (1987) *Eur. J. Biochem. 165*, 55.
Van Hoek, A., & Visser, A. J. W. G. (1985) *Anal. Instrum. 14*, 359.
Van Hoek, A., & Visser, A. J. W. G. (1990) *Appl. Optics 29*, 2661.
Zidovetzki, R., & Lester, D. S. (1992) *Biochim. Biophys. Acta 1134*, 261.