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The use of the fluorescence properties of indolocarbazole analogs of adenosine triphosphate as probes of the activating conformational change in protein kinase C☆

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Abstract

The fluorescence properties of the protein kinase C (PKC) inhibitors staurosporine, a naturally occurring indolocarbazole, and the synthetic compound 12-(2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5*H*-indolo[2,3-*a*]pyrrolo[3,4-*c*]carbazole (GÖ 6976), which share the indolo[2,3-*a*]pyrrolo[3,4-*c*]carbazole core, were used to probe the adenosine triphosphate (ATP)-binding site of PKCα. Upon binding to PKCα, the fluorescence emission of staurosporine and GÖ 6976 increased in intensity and the maxima shifted slightly to shorter wavelengths. For staurosporine, while the fluorescence lifetime was relatively insensitive to the PKC activation state, a long rotational correlation time appeared, consistent with a more restricted motion within the ATP-binding site. This effect was obtained only in the presence of the activators of the enzyme diacylglycerol or phorbol ester along with calcium and was obtained in spite of the inhibition of phosphorylation of a peptide substrate by the effective blocking of the ATP-binding site by the probe. This shows that the catalytic domain of PKC, within which the ATP-binding site resides, changes conformation upon enzyme activation. The results demonstrate the usefulness of indolocarbazole-analogs in probing ATP-binding site conformational changes and show that the ATP-binding site within the PKC catalytic domain participates in the activating conformational change in the enzyme.

Keywords: Indolocarbazoles; Fluorescence spectroscopy; Protein kinase C

1. Introduction

Protein kinase C (PKC) family of enzymes are key elements in the control of numerous cell pathways that act as molecular switches by catalyzing the phosphorylation of diverse target proteins, thereby regulating function [1–4]. The PKC isoforms are comprised of the "conventional" Ca^{2+}- and diacylglycerol-dependent(cPKC) α, βI, βII, γ, the "novel" Ca^{2+}-independent (nPKC) δ, ε, η, θ, and the "atypical" Ca^{2+}- and diacylglycerol-independent (aPKC) ζ, ι, λ isoenzymes. In order for the PKC isoenzymes to locate to the target, it interacts with a "docking surface" such as the lipids in the surface of the cell membrane or with a peptide sequences in target proteins such as filamentous-actin [5] or signaling molecules in the cell such as RhoA [6]. The docking and activation processes are distinct events the detail of which are still being determined.

In a series of papers from this laboratory, we characterised the molecular details of the interaction of PKC with membranes, filamentous-actin and the small GTPase RhoA [5–12]. The binding and activation of PKC are distinct processes which in the cPKC isoforms have different Ca^{2+}-concentration dependencies. For PKCα, translocation to a membrane occurs at low 1–10 μM Ca^{2+} whereas an *activating conformational change* in the enzyme activation occurs only at higher Ca^{2+} levels [12]. Here, it was shown that the fluorescent compound sapintoxin D, a member of a class of highly potent and specific PKC-activators known as phorbol esters involved in tumor-promotion, has distinct motional properties according to the Ca^{2+}-concentration-induced activation state of the enzyme [12]. The correlation time of sapintoxin D increased from 10–12 to ∼40 ns upon addition of Ca^{2+}

Abbreviations: C6-NBD-PS: 1-palmitoyl, 2-7-nitrobenz-2-oxa-1, 3-diazol-4-yl- (NBD-) hexanoyl phosphatidyl serine; DAG: dioleoylglycerol; DMSO: dimethyl sulfoxide; GÖ 6976: (12-(2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5*H*-indolo [2,3-*a*]pyrrolo[3,4-*c*]carbazole; LUV: large unilamellar vesicles; MBP_{4-14}: myelin basic protein peptide; PKC: protein kinase C; POPC: 1-palmitoyl, 2-oleoyl phosphatidylcholine; TPA: 4b-12-*o*-tetradecanoylphorbol-13-acetate

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(and appearance of activity) and increased further to over ~80 ns in the presence of cholesterol and PE which elevate activity. There was a strong correlation between a slower rotational correlation time for the phorbol ester in its binding pocket and increased activity of the enzyme. The extent to which the activating conformational changes are felt in the ATP-binding site in the catalytic domain of PKC as a function of the activation state of the enzyme have remained to be investigated.

There are a number of fluorescent compounds that are also highly specific inhibitors of PKC. These include indolocarbazoles the most commonly used example being the naturally occurring compounds staurosporine [13,14] (see Fig. 1) and a series of synthetic analogs such as 12-(2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5*H*-indolo[2,3-*a*]pyrrolo[3,4-*c*]carbazole (GÖ 6976) [15] members of a large number of other bisindolylmaleimides that have been described [16,17] some rhodamine and fluorescein derivatives of which have been utilized as fluorescent probes of PKC [18,19]. To date, apart from the studies in this laboratory on sapintoxin binding to the C1-phorbol ester binding domain [12], there have been no time-resolved fluorescence studies of fluorescent activators or inhibitors of PKC. The indolocarbazoles are of additional interest since their action against PKC has anticancer [20,21], and anti-fungal and anti-hypertensive therapeutic potential [22].

In this study, the spectral and steady-state properties of fluorescence indolocarbazole ATP-analogs staurosporine and GÖ 6976 (see Fig. 1) were assessed for the ability to provide information on the ATP-binding site on the catalytic domain of PKC and potentially kinase in general. The fluorescence yield of staurosporine is superior to GÖ 6976 and was used for further time-resolved measurements. Upon activation of PKCα there was a spectral emission blue shift and an increase in fluorescence yield, lifetime and steady-state anisotropy along with a longer rotational correlation time of staurosporine that coincided with the activating conformational change in the enzyme.

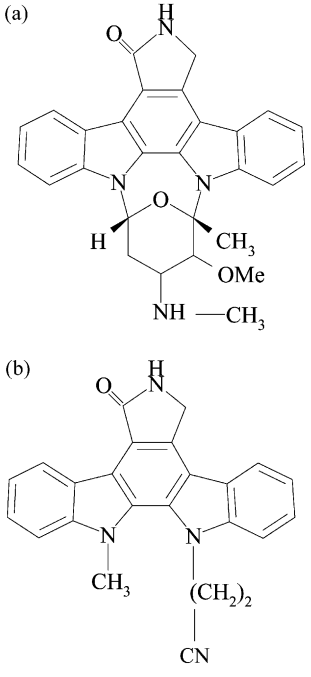

Fig. 1. Molecular structures: (a) staurosporine, and (b) GÖ 6976, a synthetic compound (12-(2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5*H*-indolo[2,3-*a*]pyrrolo[3,4-*c*]carbazole).

2. Experimental

2.1. Materials

Staurosporine and GÖ 6976 were obtained from Calbiochem (San Diego, CA). TPA was from Sigma (St. Louis, MI), ATP was from Boehringer Mannheim (Indianapolis, IN) and (γ-^{32}P) ATP was from DuPont NEN (Boston, MA). All the phospholipids were from Avanti Polar Lipids (Alabaster, AL). The phospholipid stock concentrations were verified by phosphorus assay [23]. Myelin basic protein peptide substrate (MBP_{4-14}: QKRPSQRSKYL) was custom synthesized by the Protein Chemistry Facility of the Jefferson Cancer Institute (Philadelphia, PA). Solvents used in this study were of analytical grade, obtained from Fisher Scientific (Pittsburgh, PA) or Sigma (St. Louis, MI) and used without further purification.

2.2. Expression and purification of PKC and RhoA

Recombinant PKCα was prepared using the baculovirus *Spodoptera frugiperda* (Sf9) insect cell expression system and purified to homogeneity as described elsewhere [24] with modifications [25]. Recombinant protein RhoA was expressed in *Escherichia coli* from a pGEX-2T vector (kindly provided by Dr. Ridley, Ludwig Institute for Cancer Research, London, UK) as a glutathione S-transferase fusion protein and purified as described previously [26]. Protein concentrations were estimated using a protein assay kit (Bio-Rad).

2.3. Preparation of lipid vesicles

Large unilamellar vesicles (LUV) were prepared as previously described [27]. Briefly, aliquots of the required phospholipids in chloroform were placed in a test tube and the solvents removed under a stream of nitrogen. A volume of 50 mM Tris–HCl, pH 7.4 was then added to give a total lipid concentration of 1 mM. After vortexing, the sample was passed through a 0.1 μm polycarbonate filter using the Avestin Liposofast Extruder (Avestin, Ottawa, Canada). The vesicles consisted of 1-palmitoyl, 2-oleoyl phosphatidylcholine (POPC) and brain phosphatidylserine (BPS) at 4:1 molar ratio with dioleoylglycerol (DAG) (4 mol% of total lipids) or 1 μM 4β-12-*o*-tetradecanoylphorbol-13-acetate (TPA), the final lipid concentration being 50–100 μM as required. In this work, either DAG or the phorbol ester TPA

were used interchangeably. The latter is useful for experiments performed without vesicles, as with RhoA, since it can be dispersed in aqueous solutions unlike DAG which must be co-dispersed in the lipid bilayer.

2.4. PKC activity

The activity of PKCα was determined, using MBP_{4-14} as a substrate, in a lipid vesicle- or RhoA-dependent assay. The assay system consisted of 50 mM Tris–HCl (pH 7.4) containing 0.1 mM Ca^{2+}, 50 μM MBP_{4-14} and either lipids (150 μM) or RhoA (50 nM). After thermal equilibration to 30°C, assays were initiated by the simultaneous addition of PKCα (0.3 nM) along with a solution containing 5 mM Mg^{2+}; 15 μM ATP; 0.3 μCi (γ-^{32}P) ATP (3000 Ci/mmol). The desired amount (50 or 100 nM) of staurosporine or GÖ 6976 was added from a dimethyl sulfoxide (DMSO) stock. The final assay volume was 75 μl. The reaction was terminated after 30 min with 100 μl of 175 mM phosphoric acid. Following this, a 100 μl aliquot was transferred to Whatman P81 filter papers, which were washed three times in 75 mM phosphoric acid before radioactivity associated with the paper, i.e. the phosphorylated peptide, was determined by liquid scintillation counting.

2.5. Fluorescence spectroscopy

Fluorescence data was collected for 2 ml sample volumes, with constituents added as for activity measurements but with MBP_{4-14} and ATP omitted, using an SLM 48000 multifrequency phase-modulation fluorimeter. Steady-state spectra and anisotropy were measured using a xenon lamp as the excitation source set at 335 nm for both staurosporine and GÖ 6976. For anisotropy measurements emission was collected through 375 nm redpass filters and Glan-Thompson polarisers set at the desired orientation. To assess PKC–membrane association, the increase in the steady-state anisotropy of 1-palmitoyl, 2-7-nitrobenz-2-oxa-1, 3-diazol-4-yl- (NBD-) hexanoyl phosphatidyl serine (C6-NBD-PS; co-dispersed with the lipids at 1 mol% of the lipid concentration) was used as previously described [12]. Excitation was at 460 nm and emission was collected through a 530 redpass filter. For time-resolved fluorescence measurements of staurosporine excitation was obtained from a Liconix He:Cd laser (model 4240NB, 325 nm), sinusoidally modulated by a RF frequency from 10 to 120 MHz. Both steady-state and time-resolved anisotropy measurements were set in the T-format configuration. The principle and operational details were as published elsewhere [28,29]. An aqueous solution of rabbit liver glycogen was used as a reference light scatterer for fluorescence lifetime measurements. The experimental errors used in fluorescence lifetime data analysis were the averaged errors over the range of frequencies collected in data acquisition, usually ∼0.2° in the phase and ∼0.002° in the modulation. For time-resolved fluorescence anisotropy, the averaged experimental errors were ∼ 0.3° in the phase and ∼0.003° in the modulation. For fluorescence measurements the PKC and RhoA concentrations were 65 and 220 nM, respectively.

Solvent dielectric constants were obtained by mixing various amount of dioxane with water. The dielectric constant values from various ratios of dioxane/water (v/v) were taken from the literature [30].

Steady-state anisotropy r_s was calculated using the equation: $r_s = (I_{VV} - GI_{VH})/(I_{VV} + 2GI_{VH})$, where G is the instrumental correction factor, $G = I_{HV}/I_{HH}$, I_{VV} is the emission intensity with vertically polarized excitation and emission, I_{VH} is emission intensity with vertically polarized excitation horizontally polarized emission.

Phase differential data from time-resolved anisotropy measurements were analyzed using the 48000S software provided by Jobin Yvon Horiba (Edison, NJ). The data were fitted to analytical forms and compared on the basis of χ^2 and residuals. It was found that a double exponential decay was required for fluorescence lifetime analyses. For the time-resolved anisotropy, based on residuals and χ^2, the most appropriate model for staurosporine was: $r(t) = r_0[f_1 \exp(-\tau/\phi_1) + f_2 \exp(-\tau/\phi_2)]$, where r_0 is the limiting anisotropy, ϕ_1 and ϕ_2 are the rotational correlation times, f_1 and f_2 are the fractional intensities associated with the respective rotational correlation time, τ is the fluorescence lifetime.

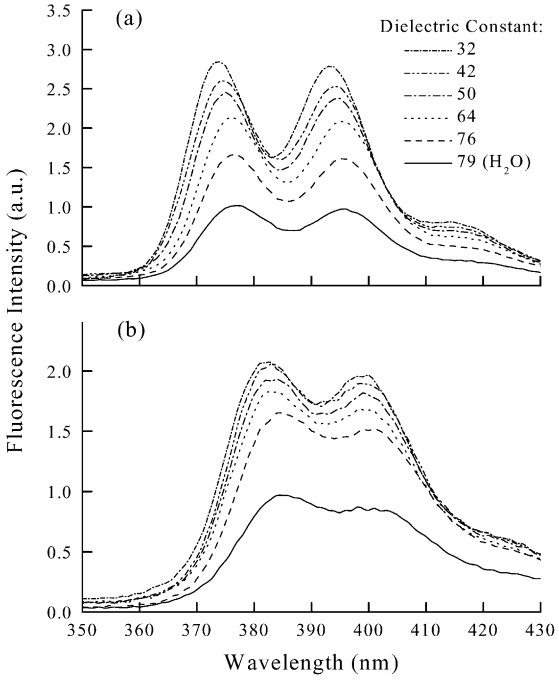

Fig. 2. Fluorescence spectra of staurosporine and GÖ 6976 in solvents of varying dielectric constant: (a) staurosporine, and (b) GÖ 6976. Solvents had varying dielectric constants obtained using dioxane/H_2O mixtures (from [30]). The excitation wavelength was set at 335 nm. Both wavelength and maximum intensity shifted with the dielectric constant. Details are as described in Section 2.

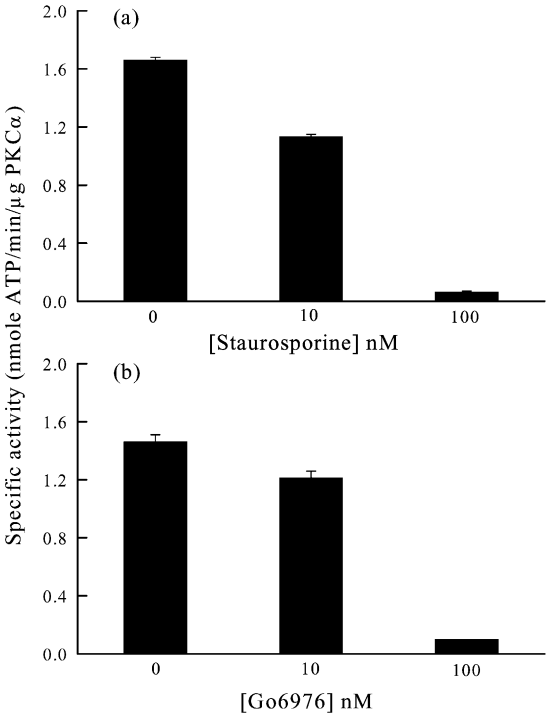

Fig. 3. Effect of staurosporine and GÖ 6976 on PKCα activity. Inhibition of PKCα activity by staurosporine (a), and GÖ 6976 (b). Details are as described in Section 2.

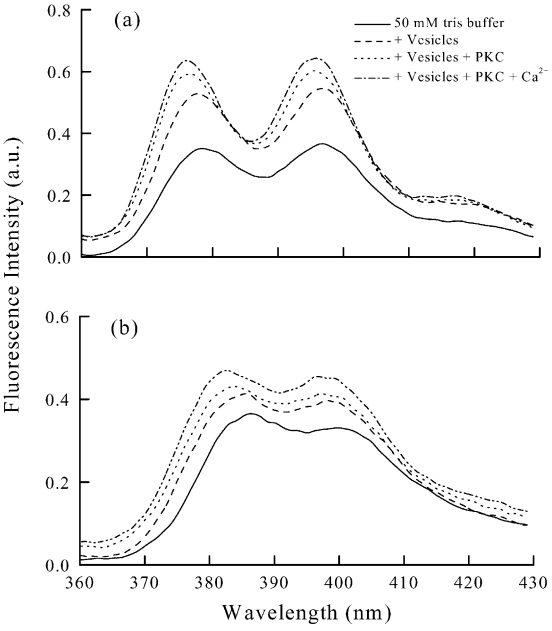

Fig. 4. Fluorescence spectra of staurosporine and GÖ 6976 bound to PKCα. Fluorescence emission spectra obtained upon excitation 335 nm for staurosporine (a), and GÖ 6976 (b), upon interaction with PKCα (65 nM) in the presence of membrane lipid vesicles (100 μM POPC/BPS, 4:1 molar with DAG at 4 mol% of total lipids) and 100 μM Ca^{2+}. Details are as described in Section 2.

3. Results and discussion

The steady-state excitation and emission spectra of staurosporine and GÖ 6976 in aqueous and other solvents is shown in Fig. 2. using various dioxane/water mixtures. The emission maxima shift to lower wavelengths with decreasing dielectric constant for GÖ 6976 while with staurosporine the effect reversed at a dielectric constant of 42. The solvent sensitivity suggests that these molecules may be useful as sensors of the environment within the ATP-binding pocket of PKC. The inhibitory effect of these compounds on PKC activity is shown in Fig. 3.

The effect of addition of Ca^{2+} (inducing translocation of PKC to the membrane) followed by addition of POPC/BPS (2:1 molar) unilamellar vesicles containing the PKC activator DAG (4 mol%) is shown in Fig. 4. The spectral maxima shifts to lower wavelengths and there is an increase in

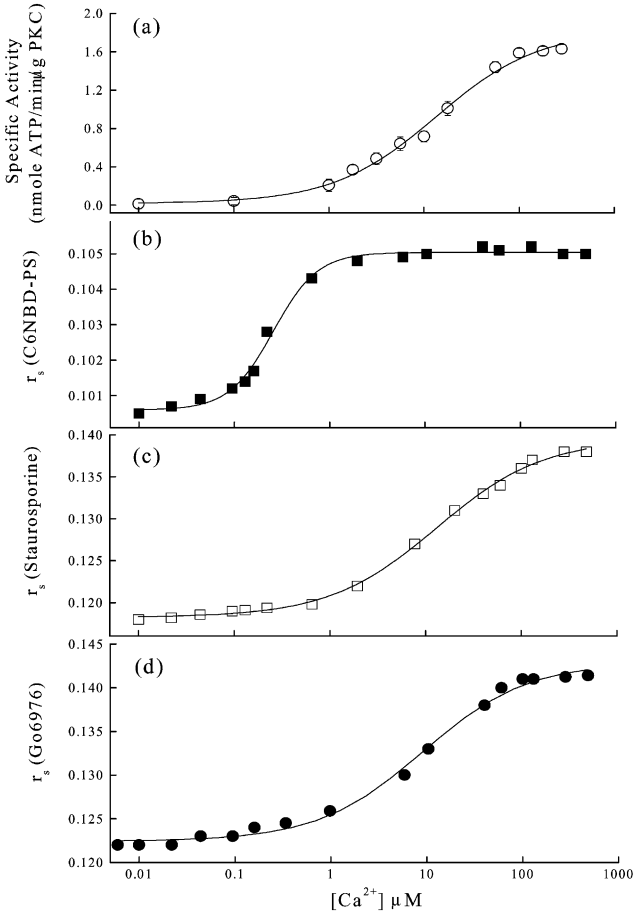

Fig. 5. Staurosporine and GÖ 6976 steady-state fluorescence anisotropy increases upon Ca^{2+}-dependent activation rather than membrane association: (a) PKCα activity, (b) the steady-state anisotropy (r_s) of C6-NBD-PS (increased anisotropy indicates PKC binding to the membrane lipids (from previously published data [12]), (c) the steady-state anisotropy of staurosporine, (d) the steady-state anisotropy of GÖ 6976. The staurosporine and GÖ 6976 curves share the same Ca^{2+}-concentration-dependent dependency as PKC activation rather than membrane binding (as shown by C6-NBD-PS anisotropy). Details are as described in Section 2.

fluorescence yield that occurred with both staurosporine and GÖ 6976.

To further investigate the staurosporine and GÖ 6976 environment in the PKC–ATP-binding site, the steady-state emission anisotropy was monitored as a function of the presence of Ca^{2+} and vesicles with the PKC activator DAG. Upon addition of PKC (without lipids or Ca^{2+}) the fluorescence anisotropy of both staurosporine and GÖ 6976 increased showing that these molecules bind to the inactive form of PKC. Upon addition of Ca^{2+} the PKC undergoes an activating conformational change in the activator binding C1-domain of the regulatory subunit of the enzyme. With the fluorescent ATP-analogs staurosporine and GÖ 6976, a DAG- and Ca^{2+}-dependent increase in the steady-state fluorescence anisotropy was found as shown in Fig. 5(c) and (d). This suggests that the activating conformational change in PKC is transmitted to the ATP-binding pocket in the catalytic domain even though the enzyme is inhibited in terms of its ability to phosphorylate target peptides it is trapped in an "active" conformation. The possibility that the changes in the steady-state fluorescence anisotropy were due to changes in the extent of binding of PKC to the membrane and not to changes in staurosporine or GÖ 6976 interaction with PKC cannot be discounted without recourse to time-resolved measurements.

Further investigation of the Ca^{2+}-dependence of the fluorescence anisotropy increase is shown in Fig. 5(a). The translocation of PKC to membranes causes a perturbation of the lipid bilayer that can be monitored by measurements of the fluorescence anisotropy of the lipid probe C6-NBD-PS [12]. The C6-NBD-PS fluorescence anisotropy increases at a low Ca^{2+} for which there is no PKC phosphorylation activity (Fig. 5(b)). Activity appears at a higher level of Ca^{2+} that is identical to the level of Ca^{2+} required for the increase in staurosporine and GÖ 6976 fluorescence anisotropy. In a previous study from this laboratory, it was shown that the fluorescence anisotropy of sapintoxin D, a fluorescent phorbol ester and activator that binds to the C1-domain, likewise increased at the same Ca^{2+} level that produced activity. From this it may be concluded that the unfolding of PKC from its (membrane-associated) inactive form to the active conformation involves a conformational change in the enzyme in

Table 1
Fluorescence lifetimes and rotational correlation times for staurosporine upon interaction with PKCα[a]

	Addition	Model	τ_1	f_1	τ_2	f_2		χ^2
Fluorescence lifetimes								
(i)[b]	–	1τ	4.32	1.00				75.48
		2τ	**5.56**	**0.80**	**1.31**	**0.20**		**1.12**
(ii)[b]	Vesicles	1τ	5.02	1.00				53.07
		2τ	**5.76**	**0.88**	**1.45**	**0.12**		**0.92**
(iii)[b]	Vesicles + PKCα + Ca^{2+}	1τ	5.34	1.00				95.23
		2τ	**6.77**	**0.86**	**1.82**	**0.14**		**1.27**
(iv)[c]	PKCα	1τ	4.66	1.00				428.75
		2τ	**6.08**	**0.83**	**0.96**	**0.17**		**1.84**
(v)[c]	PKCα + RhoA + TPA	1τ	4.72	1.00				489.62
		2τ	**6.42**	**0.76**	**1.29**	**0.24**		**1.71**
	Addition	Model	ϕ_1	f_1	ϕ_2	f_2	r_0	χ^2
Rotational correlation times								
(i)[b]	–	1φ	**0.50**	**1.00**			**0.35**	**0.88**
		2φ	0.50	0.92	0.49	0.02	0.35	1.02
(ii)[b]	Vesicles	1φ	0.39	1.00			0.35	3.19
		2φ	**1.98**	**0.33**	**0.16**	**0.71**	**0.35**	**0.56**
(iii)[c]	Vesicles + PKCα + Ca^{2+}	1φ	0.57	1.00			0.35	3.73
		2φ	**15.10**	**0.23**	**0.57**	**0.77**	**0.35**	**0.62**
(iv)[c]	PKCα	1φ	0.51	1.00			0.35	3.62
		2φ	**2.85**	**0.10**	**0.46**	**0.90**	**0.35**	**1.47**
(v)[c]	PKCα + RhoA + TPA	1φ	0.60	1.00			0.35	4.54
		2φ	**18.26**	**0.08**	**0.65**	**0.92**	**0.35**	**0.90**

[a] Fluorescence data was collected for PKCα (65 nM) with either vesicles (100 μM: POPC/BPS, 4:1 molar ratio with DAG at 4 mol% of total lipids) or RhoA (220 nM) and included staurosporine (50 nM), Ca^{2+} (100 μM) and TPA (1 μM) (for RhoA-dependent activity) or DAG (for vesicle-dependent activity. Details are as described in Section 2. t_1 and t_2: fluorescence lifetimes; f_1 and f_2: rotational correlation times in ns (10^{-9} s); f_1 and f_2: fractional intensities; r_0: limiting anisotropy; ns: 10^{-9} s; χ^2: reduced chi-square. Bold typeface indicates the preferred model (based on χ^2).

[b] 50 mM Tris buffer.

[c] 50 mM HEPES buffer.

the regulatory domain at the ATP-binding site where staurosporine and GÖ 6976 bind.

The steady-state fluorescence anisotropy can be used only as an approximate indication of the motional freedom of a fluorophore. Time-resolved lifetime and anisotropy are required to verify that that it is not changes in the amount of PKC–staurosporine–GÖ 6976 complex bound to the membrane but an altered PKC–staurosporine and –GÖ 6976 interactions stemming from an altered PKC-conformation that underlie the changes in fluorescence anisotropy. If the staurosporine and GÖ 6976 bind tightly in the ATP-binding pocket, one would anticipate that a characteristic rotational correlation time significantly longer than that without PKC should be recoverable from time-resolved data.

The fluorescence lifetime and rotational correlation time data for staurosporine, analyzed according to single and double exponential decays are rotational correlation times are shown in Table 1. In comparing staurosporine and GÖ 6976, the former has a greater fluorescence yield and was therefore used for time-resolved measurements. The results show that the fluorescence lifetime was relatively insensitive to the activation state of PKC. Although inactive PKC appears to associate with staurosporine a correlation time distinct from that seen with PKC could not be recovered from the analysis. By contrast, in the presence of Ca^{2+} and phorbol ester a long rotational correlation time of $\sim$15 ns appears which to be the basis for the higher steady-state fluorescence anisotropy. In addition to lipid-dependent activation, PKC can be activated by phorbol ester in the presence of the small GTPase RhoA [6], an important signaling molecule involved in cytoskeletal remodeling and other cell processes. In the presence of purified RhoA, TPA and Ca^{2+} again a long rotational correlation time for staurosporine bound to PKCα was recovered. The finding of a similar staurosporine rotational correlation time in two very different activation systems suggests that the two-correlation time model in the analysis is valid.

4. Conclusion

In conclusion, the results with fluorescent ATP-analogs suggest that while ATP binds to PKC in its inactive state its affinity greatly increases upon activation in both a membrane lipid and non-membrane lipid system. The strong affinity of the fluorescent ATP-analogs prevents ATP from binding and provides the inhibition of PKC activity. However, the activating conformational change in PKC that has been described on the basis of changes to the regulatory domain of PKC [12] appears to also occur at the ATP-binding site in the catalytic domain of the enzyme in spite of the actual lack of catalytic activity. The use of time-resolved anisotropy of fluorescent analogs of ATP may find general application in the wider fields of ATPases and kinases.

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