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Membrane–phorbol ester interactions monitored by circular dichroism

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The interaction of phorbol 12,13-dibutyrate (PDBu), 12-*O*-retinoylphorbol 13-acetate (RPA) and 12-*O*-tetradecanoylphorbol 13-acetate (TPA) with L-α-phosphatidylserine-containing small unilamellar vesicles or erythrocyte ghosts was monitored by circular dichroism (CD). No change in the CD spectra of PDBu was observed upon binding, while RPA and TPA spectra were slowly affected by the interaction. The changes in RPA and TPA spectra were assigned to the embedding of these molecules in the membrane bilayers. In the presence of 10^8 cells/ml, after one minute incubation, about 2 to 5% of the amount of phorbol ester added is embedded in the membrane. It is suggested that either phorbol esters entering the membrane is not a prerequisite for protein kinase C activation or the amount of phorbol esters necessary to activate protein kinase C is very small.

Phospholipid- and Ca^{2+}-activated protein kinase (protein kinase C) is involved in phosphorylation of many cellular proteins and regulation of many cell functions [1]. Protein kinase C is believed to be the site of action of the second messenger, diacylglycerol, generated in vivo by the breakdown of inositol phospholipids [2]. Diacylglycerol is thought to reduce the Ca^{2+}-requirements of the enzyme to physiological concentrations. Phorbol esters similarly activate protein kinase C [3,4].

Although they may bind to the same site, diacylglycerol and phorbol esters differ upon the following point: whereas diacylglycerol is endogenously generated at the plasma membrane in response to extracellular signal, the phorbol esters are able to incorporate the internal membranes of the cell as well.

Full understanding of protein kinase C (PKC) activation requires knowledge of the complex structure which generates kinase activity. To describe this structure, it is necessary to characterize the various physical interactions involving this enzyme and its activators. New insights were recently obtained with PKC-containing model membranes [5–8]. It appeared that the amount of phospholipids required for maximum protein kinase C activation always exceeded that required for PKC-membrane binding. Consequently, it was proposed that additional phospholipids may be required for substrate binding. On the other hand Bazzi and Nelsestuen have pointed out that protein kinase C activity measurements are performed in complex aggregated and that substrate and phospholipid requirements for activity may be related to properties of these aggregates [7,8]. An important problem is the slow dynamics of aggregation, since the molecules in the aggregates may not be in equilibrium with molecules in solution on the time scale of the assay.

These observations prompted us to study the interactions of several phorbol esters with synthetic and natural membranes. In this paper, we report the study of the kinetics of interaction of phorbol 12,13-dibutyrate and 12-*O*-tetradecanoylphorbol 13-acetate with L-α-phosphatidylserine-containing small unilamellar vesicles and erythrocytes ghosts. 12-*O*-Retinoylphorbol 13-acetate

Abbreviations: PDBu, phorbol 12,13-dibutyrate; RPA, 12-*O*-retinoylphorbol 13-acetate; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; PKC, protein kinase C; PC, phosphatidylcholine; PS, phosphatidylserine; SUV, small unilamellar vesicles; CD, circular dichroism; DMSO, dimethyl sulfoxide.

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was also considered, the hydrophilic tail of which is strongly absorbing around 350 nm and may constitute an interesting probe. The modifications of the spectral pattern of these three compounds through interaction with membrane were also thoroughly examined as far as this could yield valuable informations concerning the localization of the different regions of the molecules.

Materials and Methods

Phorbol 12,13-dibutyrate (PDBu), 12-*O*-tetradecanoylphorbol 13-acetate (TPA), 12-*O*-retinoylphorbol 13-acetate (RPA), L-α-phosphatidylserine (PS) from bovine brain and L-α-phosphatidylcholine (PC) from egg yolk type V-E were purchased from Sigma Chemical Co. All other reagents were of the highest quality available, and deionized, double-distilled water was used throughout the experiments. Unless otherwise stated, phosphate-buffered saline (PBS) solutions were 150 mM NaCl, 5 mM sodium phosphate at pH 7.3.

Absorption spectra were recorded on a Cary 219 spectrophotometer and circular dichroism (CD) spectra on a Jobin Yvon Mark V dichrograph. In the Figs. 1–3 $\Delta\epsilon$ is the differential molar dichroic absorption coefficient ($cm^{-1} \cdot M^{-1}$). The spectra of the suspensions of vesicles without phorbol ester were substracted from those with phorbol ester. They consist of a plain curve of increasing $\Delta\epsilon$ with decreasing wavelengths, originating from the light scattering of vesicles. The absence of artefact due to light diffusion in the CD study was ascertained by checking that the CD spectral pattern depended neither on the cuvette path length (varied for this control between 0.02 cm and 0.2 cm), nor on the distance between the sample cell and the detector (varied between 0 and 10 cm from the end-window of the photomultiplier tube).

Unilamellar phospholipid vesicles. Small unilamellar vesicles (SUV) were prepared according to Newman and Huang [9]. Sonication was performed at room temperature and under nitrogen. PS-containing SUV were prepared with PC and PS in the molar ratio 4 : 1.

Erythrocyte ghosts. Erythrocytes, drawn from human healthy donors of the Seine Saint Denis (France) blood bank and collected in heparinised tubes, were washed three times via $1000 \times g$ centrifugation in phosphate-buffered saline and the buffer coat discarded after each centrifugation. Packed erythrocytes were hemolyzed in a 50-fold volume of 5 mM sodium phosphate buffer (PBS, pH 7.4). Membranes were sedimented by centrifugation at $16000 \times g$ for 10 min and washed twice in 5 mM sodium phosphate buffer. Protein concentration was determined according to Lowry procedure [10]; 10^{10} cells yielded a mean of 4.6 ± 0.6 mg protein (10 experiments).

Unless otherwise stated an aliquot of PDBu, TPA and RPA in ethanol was added to the appropriate solvent in order to obtain 50 μM phorbol ester solutions. The experiments were performed at 37 °C.

Results

Absorption and CD spectra of PDBu, TPA and RPA

In order to determine the origin of the bands observed, we first analyzed the absorption and CD spectra of these compounds in various solvents: PBS buffer, ethanol and dimethyl sulfoxide (DMSO). The spectral data obtained are reported in Table I.

Small aliquots of stock-solutions in ethanol ($7.3 \cdot 10^{-3}$ M RPA or $8.1 \cdot 10^{-3}$ M TPA) were added to the different solvents studied. In DMSO, due to the strong absorption of this solvent, it was not possible to obtain information at wavelengths lower than 250 nm. In PBS buffer, due to its strong hydrophobicity, TPA aggregated immediately after the addition of the aliquot of the stock-solution; a strong Duysens effect flattened the spectra [11]. In contrast, RPA aggregated more slowly and it was possible to record its spectrum within the minutes which preceded its high aggregation state.

The absorption and CD spectra of TPA and RPA in ethanol are shown in Fig. 1. Between 200 and 300 nm the absorption and CD spectra of the three compounds are similar: CD spectra exhibit two bands, hereafter labelled I and II around 230 and 270 nm, respectively. In the absorption spectra a strong band correspond to the CD band I whereas there is no detectable absorption band corresponding to the CD band II. Between 300 and 450 nm TPA and PDBu exhibit similar spectra with a negative CD band (III) around 340 nm; no

TABLE I

Absorption and CD data for phorbol ester in various solvents and in the presence of SUV or erythrocyte ghost

The wavelength of the absorption or CD maxima are indicated in nm together with the values of ϵ or ΔE. Experimental conditions are the same as in Figs. 1 and 2.

Phorbol ester	Solvent	Band I	II	III	IV
TPA	ethanol	230(7000)			
		227(+17.4)	265(−2.7)	337(−0.8)	
	DMSO		268(−2.6)	337(−0.52)	
	H_2O				
	SUV		273(+0.9)	333(+0.66)	
	ghost		282(−1.9)	329(−0.41)	
RPA	ethanol	230(7000)			359(34000)
		226(+16.5)	273(−3.2)		348(−2.4)
	DMSO		271(−3.8)		354(−1.9)
	H_2O		282(−5.1)		379(−3.1)
	SUV		276(−2.5)		359(−2.6)
	ghost		284(+2.7)		360(−4.4)
PDBu	DMSO		270(−2)	340(−0.5)	
	H_2O		268(−3)	328(−1)	

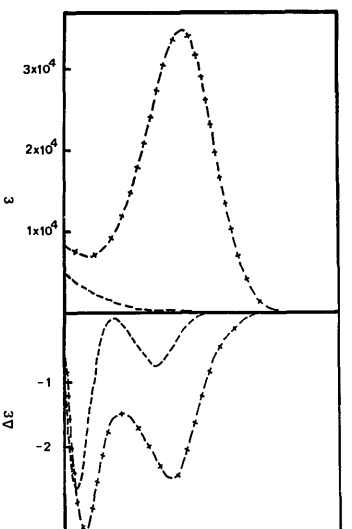

Fig. 1. Absorption (upper) and circular dichroism (lower) spectra of TPA (- - - - -) 10^{-4} M and RPA (+ ——— +) $3 \cdot 10^{-4}$ M in ethanol.

absorption band is detectable in that region. RPA exhibits quite different spectra with a strong absorption band around 360 nm to which correspond a negative CD band (IV). Bands I, II, III are due to transitions of the chromophore common to the three compounds, i.e., the phorbol entity. Band I can be assigned to a $\pi \rightarrow \pi^*$ transition whereas bands II and III, which are detectable in CD but not in absorption, can be assigned to $n \rightarrow \pi^*$ transitions. Band IV which is observed in RPA only is due to $\pi \rightarrow \pi^*$ transition of the retinoate chromophore; retinoic acid exhibits similar absorption band around 350 nm but is not optically active. Band III is not detectable in the CD spectrum of RPA and it is likely that it is masked by band IV.

Interaction of PDBu, TPa and RPA with PS-containing SUV

It has been shown that, in the presence of Ca^{2+}, PS always activates protein kinase C more than any other negatively charged phospholipids. Phosphatidylcholine is unable to activate protein kinase C [12–14]. For these reasons we used PS-containing SUV in the following experiments.

The PDBu spectrum in PBS buffer was not modified by the addition of PS-containing SUV, within the limits of accuracy. In contrast the spectrum of RPA was progressively modified by the addition of PS-containing SUV at a molar ratio RPA : PS = 1 : 2. The dichroic band at 379 nm shifted to 359 nm and increased in intensity (Fig. 2). The modification of the signal, monitored at 350 nm (Fig. 3), was slow ($t_{1/2} = 18$ min).

The spectra of aggregated TPA was also modified by the addition of PS-containing SUV: a negative band (band III) appeared at 330 nm (Fig. 2). As can be seen in Fig. 3, its appearance was more rapid ($t_{1/2} = 2$ min) than the modification of the 350 nm RPA band. A similar spectrum was obtained when the experiment was performed with PC SUV. However, in this case, the kinetics of the interaction was slower ($t_{1/2} = 5$ min) and the signal was less stable.

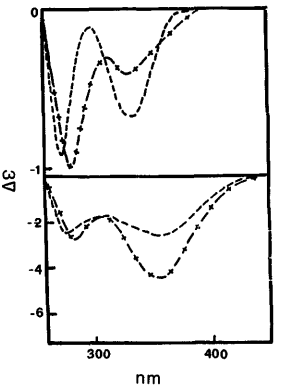

Fig. 2. Circular dichroism spectra of TPA (upper) and RPA (lower) in the presence of SUV (- - - - -) and erythrocyte ghosts (+ ——— +) after 2 h incubation at 37 °C. Experimental conditions: PBS buffer, 50 μM TPA, 50 μM RPA; PS- and PC-containing SUV corresponding to 0.5 mM phospholipid at a molar ratio of PS/PC equal to 1 : 4; protein concentration in erythrocyte ghosts was 0.05 g/l.

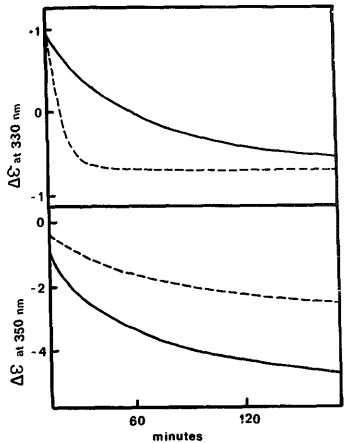

Fig. 3. Kinetics of interaction of TPA (upper) and RPA (lower) with SUV (- - - - -) and erythrocyte ghosts (———). The kinetics were monitored using the variation of $\Delta\epsilon$ at 330 nm and 350 nm for TPA and RPA, respectively. Experimental conditions are the same as in Fig. 2.

Using the data of Fig. 3, the initial rates of the phorbol ester–membrane interactions have been calculated. v_i values equal to 3.2 $\mu M \cdot min^{-1}$ and 0.44 $\mu M \cdot min^{-1}$ have been obtained for TPA and RPA, respectively.

Interaction of PDBu, TPA and RPA with erythrocyte ghosts

Membranes of erythrocyte have been chosen for the reasons that they contain a high proportion of PS. This phospholipid accounts for 16% of the total molar phospholipid concentration and 70% of the negatively charged phospholipids [15]. Experiments similar to those above described were performed with ghosts. In these experiments the number of phorbol ester molecules per ghost was $2.8 \cdot 10^8$. The addition of PDBu to ghosts did not yield significant modifications of the spectral pattern of PDBu. In the case of TPA and RPA spectral modifications occurred slowly; $\Delta\epsilon$ at 330 nm and 350 nm have been plotted as a function of time to follow the kinetics of the interaction of TPA and RPA with ghosts, respectively (Fig. 3). The CD spectra obtained at the end of the kinetics are shown in Fig. 2. $t_{1/2}$ values are equal to 15 min and 30 min for TPA and RPA, respectively. Using the data of Fig. 3, the initial rates of the phorbol ester–membrane interactions have been calculated. v_i values equal to 0.80 $\mu M \cdot min^{-1}$ and 1.0 $\mu M \cdot min^{-1}$ have been obtained for TPA and RPA, respectively.

Discussion

PDBu has been shown to bind to model phospholipid membranes [16] or to Triton X-100/PS mixed micelles providing the Triton X-100 concentration is sufficiently high [6]. However, we did not observe any noticeable change of PDBu spectra in the presence of PS-containing PC vesicles or erythrocyte ghosts. In contrast, a strong modification of TPA or RPA spectra was observed in the presence of these membranes, indicating definitively the existence of an interaction as already observed with Triton X-100/PS micelles [6]. In particular the embedding of the retinoate group in the lipid bilayer is evidenced by the 15 nm red shift of band IV: indeed the $\pi \rightarrow \pi^*$ transition of polyenes is well known to red shift when the solvent is changed from methanol to DMSO or membranes [17–19].

It must be noted that the band IV wavelength of RPA in buffer should theoretically be between that of methanol and that of DMSO. Its position at much higher wavelength is explained by aggregation which expels water from the environment of the chromophore as it has been already observed with other polyenes.

Under our conditions we can estimate that, after one minute incubation and in the presence of 10^8 cells/ml, 5% and 2% of the phorbol ester added have been embedded inside the membrane in the case of TPA and RPA, respectively. In the case of experiments performed with physiological amounts of phorbol ester, for instance 20 nM phorbol ester and 10^8 cells/ml, one can make the assumption that after one minute incubation, a similar proportion of phorbol ester has been incorporated inside the membrane. These data together with the observations that protein kinase C is activated by the three phorbol esters under consideration (RPA being as active as TPA (Castagna, M., unpublished data)) and that this activation is a rapid process which does not appear to increase with time of incubation, led us to suggest two possibilities: (i) the deep embedding of phorbol ester inside the membrane is not essential for protein kinase C activity, (ii) the embedding of phorbol ester is necessary for protein kinase C activity but the quantity required is very low and the small amount which has entered the cell after one minute is enough to activate protein kinase C.

Acknowledgments

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