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INFLUENCE OF DI- AND TRI-PHENYLETHYLENE ESTROGEN/ANTIESTROGEN STRUCTURE ON THE MECHANISMS OF PROTEIN KINASE C INHIBITION AND ACTIVATION AS REVEALED BY A MULTIVARIATE ANALYSIS

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Abstract—We have performed a systematic study of the interaction of 36 di- and tri-phenylethylene derivatives (DPEs and TPEs) with protein kinase C (PKC). The results were submitted to a multivariate analysis in order to identify the structural features that might be implicated in interference with the activity of three PKC subspecies under three enzyme activation conditions. Four groups of test-compounds, each with common chemical features, could be distinguished clearly. The first group comprised all TPEs substituted with at least one basic dialkylaminoethoxy side-chain. These inhibited type α, β and γ PKC subspecies activated by Ca^{2+} and phosphatidylserine (PS) with or without diolein (DO) at micromolar concentrations but did not inhibit protamine sulfate phosphorylation. The other effectors, which all possessed a 1,1-bis-(*p*-hydroxyphenyl) ethylene moiety, influenced PKC activity at high concentrations (30–200 μM) and could be divided into two groups. One group constituted PKC inhibitors in the TPE series and inhibited PKC activated by Ca^{2+}, PS and DO, as well as protamine sulfate phosphorylation. The other group constituted dual-type inhibitors/activators in the DPE series and stimulated PKC in the presence of Ca^{2+} and low PS concentrations but inhibited the enzyme in the simultaneous presence of DO. The fourth group of compounds was inactive and had, for the most part, one or two substituents with weak steric hindrance. In agreement with previous data for six lead compounds, this study suggests that, in these chemical series, a basic amino side-chain leads to interaction with phospholipid and the regulatory domain of PKC, whereas a 1,1-bis-(*p*-hydroxyphenyl) ethylene moiety leads to interaction with the catalytic domain of the enzyme.

Protein kinase C (PKC**), a Ca^{2+}-activated and phospholipid-dependent protein kinase, is found in many tissues and exists as a large family of multiple subspecies (reviewed in Ref. 1). The limited proteolysis of PKC by the Ca^{2+}-dependent neutral protease calpain has revealed two functional domains, a regulatory domain and a protein kinase (or catalytic) domain. The protein kinase domain is fully active in the absence of effectors [2] and contains an ATP-binding sequence [1]. The regulatory domain contains the sites of interaction of Ca^{2+}, phospholipid, diacylglycerol and tumour-promoting phorbol esters which bind and activate the enzyme [3].

It is generally accepted that PKC plays a crucial role in cell surface signal transduction. PKC is implicated in cellular functions such as membrane conductance, interaction and down-regulation of receptors, gene expression, control of growth and differentiation; and in several physiological functions including the release, secretion and exocytosis of cellular messengers from a variety of endocrine, exocrine and neuronal tissues, and the modulation of smooth muscle contraction (reviewed in Refs 1, 4 and 5). PKC subspecies possess slightly different biochemical properties and are expressed in different proportions within different tissues and cell types [1, 6–8]. This suggests that they may have distinct functions in the processing and modulation of response and that their effectors, apart from being potential tools in fundamental studies, may display a gamut of activities in a variety of systems (central nervous system, endocrine and immune systems, skin, bone, etc.).

Many derivatives of diphenylethylene (DPE) and triphenylethylene (TPE) are well known for their estrogen, antiestrogen and/or antitumoral action [9–

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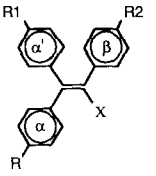
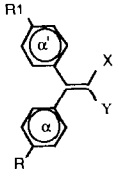
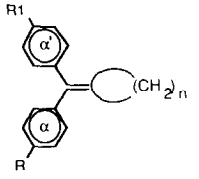
** Abbreviations: CFA, correspondence factorial analysis; DPE, 1,1-diphenylethylene derivative; DMSO, dimethyl sulfoxide; DO, diolein; E_2, 17-β estradiol; ER, estrogen receptor; PKC, protein kinase C; PKM, protein kinase M; PS, phosphatidylserine; Tam, tamoxifen; OHTam, 4-hydroxytamoxifen; TPE, triphenylethylene derivative.

Table 1. Structures of DPE and TPE derivatives



TPE	X	R	R1	R2	TPE	X	R	R1	R2
1	CN	—	—	—	**13*Z***	CN	DEAE	OH	—
2*E*	CN	—	OH	—	**14**	CN	DEAE	DEAE	—
2*Z*	CN	OH	—	—	**15**	CN	—	—	DEAE
3	CN	—	—	OH	**16**	CN	DMA	DMA	—
4	CN	OH	OH	—	**18**	CN	3DMAM*	3DMAM*	—
5*E*	CN	—	OH	OH	**19**	CN	OMe	OMe	—
5*Z*	CN	OH	—	OH	**20**	CN	OiAm	OiAm	—
6	CN	OH	OH	OH	**21**	CN	OH	OH	DEAE
7*E*	CN	Me	OH	—	**22**	CN	—	—	DIAE
7*Z*	CN	OH	Me	—	**23**	CN	DIAE	DIAE	—
8	CN	OH	OH	Me	**24**	CH_2NH_2	OH	OH	—
9*E*	CN	Me	OH	OH	Tam *E*	Et	DMAE	—	—
9*Z*	CN	OH	Me	OH	Tam *Z*	Et	—	DMAE	—
11*E*	CN	OH	OiPr	—	OHTam *E*	Et	DMAE	OH	—
11*Z*	CN	OiPr	OH	—	OHTam *Z*	Et	OH	DMAE	—
13*E*	CN	OH	DEAE	—					





DPE	X	Y	R	R1	DPE	n	R	R1
25	H	iPr	OH	OH	**27**	4	OH	OH
26	Cl	iPr	OH	OH	**28**	5	OH	OH
					29	6	OH	OH

Substitutions are in para position (* indicates a hydroxy group in para). Me = CH_3; Et = C_2H_5; OiPr, $OCH(CH_3)_2$; DEAE, $OCH_2CH_2N(C_2H_5)_2$; DMA, $N(CH_3)_2$; DMAM, $CH_2N(CH_3)_2$; OiAm, $OCH_2CH_2CH(CH_3)_2$; DIAE, $OCH_2CH_2N[CH(CH_3)_2]_2$.

12]. Micromolar concentrations of TPE can inhibit PKC activity [13–16], as well as PKC-mediated signal transduction in human neutrophils [17], but the mechanism of inhibition remains unclear. We have shown recently that four DPEs and two TPEs, influence rat brain PKC activity within this concentration range via multiple mechanisms involving the regulatory and/or catalytic domains of the enzyme and/or the action of lipid [18–20]. On the basis of this improved understanding, and using these DPEs and TPEs as lead compounds, we have now investigated the capacity of an enlarged series of 36 molecules, substituted with hydroxy, bulky hydrophobic or basic amino substituents in the para position of the phenyl rings (Table 1), to modulate PKC activity. We have kept to discrete stepwise modifications in chemical structure in order to discern the influence of each substitution on PKC activity and have investigated three subfractions (I, II and III), purified from rat brain by hydroxyapatite column chromatography, that correspond to the enzymes encoded by γ-, β (βI and βII)- and α-cDNA clones, respectively [21]. Moreover, three enzyme activation conditions were tested in order to obtain information on the mechanisms of PKC modulation. The results were submitted to a multivariate analysis and revealed, in particular, that in these chemical series a 1,1-bis-(*p*-hydroxyphenyl) ethylene moiety leads to interaction with the catalytic domain of the enzyme, whereas a basic amino side-chain leads to interaction with phospholipid and the regulatory domain of the enzyme.

MATERIALS AND METHODS

Chemicals

The preparation of the DPEs and TPEs (Table 1) and their analytical characteristics are given elsewhere [9, 11, 22–24]. Tamoxifen and hydroxytamoxifen isomers were kind gifts from Dr A. H. Todd (ICI, Macclesfield, U.K.). The isomeric purity

of the test-compounds was checked by HPLC analysis as described previously [9].

Phosphatidylserine and diacylglycerol cofactors for PKC were from Serdary Research Laboratories (London, ONT, Canada). [γ-^{32}P]ATP was obtained from New England Nuclear (Dreieich, F.R.G.). Protamine sulfate (from herring) was purchased from the Sigma Chemical Co. (Poole, U.K.) and calf thymus H1 histone was prepared by the method of Oliver *et al.* [25].

Enzyme purification

PKC was purified from the rat brain soluble fraction and separated by hydroxyapatite column chromatography into three types (types I, II and III) as described previously [26]. The catalytically active fragment (PKM) was prepared by cleavage of PKC by a Ca^{2+}-dependent neutral protease (calpain) followed by isolation by DEAE-column chromatography as also described previously [27].

Assay of PKC

Unless otherwise indicated, the standard reaction mixture (0.125 mL) contained Buffer A (20 mM Tris–HCl at pH 7.5), 10 μM [γ-^{32}P]ATP (300–400 cpm/pmol), 5 mM magnesium acetate, 0.01 mM EGTA and 0.01 mM EDTA (from enzyme fraction), 0.1% glycerol (v/v), 200 μg/mL calf thymus H1 histone, 0.1 mM $CaCl_2$ and 2 μg/mL phospholipid [phosphatidylserine (PS)], with or without 0.2 μg/mL diacylglycerol [diolein (DO)]. TPEs were dissolved in DMSO. PS and DO were stored in chloroform solution. The PS solution was evaporated under a stream of nitrogen (when DO was used, it was mixed first with PS in chloroform). The residue was resuspended in ice-cold buffer A, vigorously mixed with a vortex mixer for 1 min and sonicated with a tip sonicator for 1 min at 0°. TPE solutions in DMSO were diluted in Buffer A and vigorously mixed (vortex) for 1.5 min with an aliquot of the sonicated PS-vesicles [final DMSO concentration in the incubation = 5% (v/v)]. The PS–TPE solution was added to the reaction mixture and the phosphorylation reaction was started by the addition of PKC (approximately 0.05 μg). The incubation was carried out for 3 min at 30° with gentle shaking (40 cycles/min). The radioactivity of acid-precipitable materials was quantified by liquid scintillation. All experiments were carried out at least twice. PKC activities were plotted as a function of the test-compound concentration. These graphs (not shown) enabled the determination of the EC_{50} and/or IC_{50} of each compound (concentration giving half the maximum activatory or inhibitory effect) and the percentage of the maximum activatory or inhibitory effect obtained at a test-compound concentration of 200 μM (100% is the value obtained in the absence of test-compound).

Assay of the Ca^{2+}- and PS-independent activity of PKC and PKM

With protamine sulfate as a phosphate acceptor, PKC exhibits full enzymatic activity in the absence of PS, diacylglycerol and Ca^{2+} [2]. This protein kinase activity was determined with 400 μg/mL protamine sulfate in the presence of EGTA (0.5 mM final concentration) instead of Ca^{2+}, PS and DO. PKM was determined under the same conditions with 200 μg/mL H1 histone instead of protamine sulfate. The TPE was added directly to the reaction mixture [the final DMSO concentration in the incubation was 5% (v/v)]. The results are expressed as described above.

Multivariate analysis

Data transformation. As regards the effect of a test-compound on a given PKC activity, the technique of "split data" was used to account for both the specificity (ability to activate or inhibit under different experimental conditions) and amplitude (per cent level) of this effect without any information loss. In other words, each column headed "per cent of effect" in Table 2 was divided into two columns corresponding to the true experimental values and to "anti-values" (not shown) obtained by subtracting the experimental values from the maximal response recorded in the column [e.g. for PKC I in the presence of PS, TPE **6** has an experimental value of 24 and an "anti-value" of 183 (i.e. 207 (DPE **26**) −24)]. After transformation of the data (distribution within a range of 1 to 100 on the basis of the values obtained for the 37 molecules listed in Table 2), the table of "split data" (not shown) was analysed as described below.

Correspondence factorial analysis (CFA). The transformed split data on the effect of 37 compounds (37 rows) on three activities (types I, II and III PKC) under three activation conditions (PS, PS+DO and protamine sulfate) expressed either as concentrations or per cent levels (i.e. 18 columns) were submitted to a CFA which transforms this multidimensional system into a series of 2D-factorial maps. (For detailed descriptions and applications of the method, see Refs 9 and 28–33.) The first map, the $\phi_1\phi_2$ factorial map, accounts for the greatest proportion of the total variance of the system, in this instance 87.6% (68.2 + 19.4%), and represents, by means of proximities, the main relationships between compounds, between the biological variables (i.e. the tests), and between compounds and tests. By splitting the values, as described above, it takes into account not only the selectivity of action of the compounds with respect to the end points but also the amplitude of action. Thus, in the $\phi_1\phi_2$ factorial map, modulation of the activity of one PKC subspecies under one activation condition will be represented by two points corresponding to the extreme (high/low) responses and by a vector passing through the origin of the axes, since the two columns: Σ (experimental values) = Σ (anti-values) have the same weight. The length of these vectors and the positions of the projections of their extremities on the ϕ_1 and ϕ_2 axes are, for our purpose, of little import. However, their relative parallelism or orthogonality reflects the closeness or independence of effector behaviour towards each activity. The positions of the test-compounds reflect their specificity (proximity to a vector) and the amplitude of their effect (the distance from the origin).

Automatic classification (minimum spanning tree). The matrix corresponding to the transformed table of split values was converted into a distance matrix

Table 2. Effect of DPE and TPE derivatives on PKC I (γ), II (β) and III (α) subspecies under different experimental conditions

	Effector concentration 50% (IC_{50} or $^*EC_{50}$) (μM)									Per cent of effect								
	PS			PS + DO			Protamine sulfate			PS			PS + DO			Protamine sulfate		
DPE/TPE	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III
1	>100	>100	>100	>100	>100	>100	>100	>100	>100	114	98	126	95	98	106	87	96	106
2*E*	>200	>200	>200	>200	>200	>200	>200	>200	>200	107	117	107	92	108	87	87	92	90
2*Z*	>200	>200	>200	>200	>200	>200	>200	>200	>200	106	109	96	97	105	104	96	101	94
3	>200	>200	>200	>200	>200	>200	>200	>200	>200	87	91	92	95	95	113	96	78	103
4	>200	>200	>200	130	68	69	54	32	100	80	90	94	52	43	42	39	31	50
5*E*	>200	>200	>200	>200	>200	>200	>200	>200	>200	84	91	97	80	84	91	79	81	68
5*Z*	>200	>200	>200	>200	>200	>200	>200	>200	>200	82	78	94	91	82	87	78	79	86
6	63	69	70	60	66	55	44	38	63	24	35	38	22	29	32	23	20	33
7*E*	>100	>100	>100	>100	>100	>100	>100	>100	>100	106	96	119	98	100	87	97	73	111
7*Z*	>100	>100	>100	>100	>100	>100	>100	>100	>100	113	119	107	100	111	95	103	80	77
8	>200	>200	>200	190	175	130	74	94	93	88	91	84	49	44	38	33	48	46
9*E*	>200	>200	>200	>200	>200	>200	>200	>200	>200	88	119	88	80	76	68	96	91	94
9*Z*	>200	>200	>200	>200	>200	>200	>200	>200	>200	75	83	113	100	91	80	95	85	90
11*E*	69*	76*	69*	>100	>100	>100	>100	>100	>100	163	189	170	101	78	94	94	90	79
11*Z*	62*	62*	69*	>100	>100	>100	>100	>100	>100	160	183	138	99	94	101	101	95	86
13*Z*	8	3	3	4.7	2.7	3.1	>100	>100	>100	16	20	20	10	0	2	108	97	85
13*Z*	7.5	2.7	2.8	3.5	3	4	>100	>100	>100	15	21	10	9	13	1	96	104	88
14	4.6	3	2.7	1.75	1	1.3	>100	>100	>100	31	47	20	12	19	10	101	121	101
15	3.3	2.5	3.2	4.2	2.2	2.9	>100	>100	>100	23	46	43	26	27	20	107	105	96
16	>100	>100	>100	>100	>100	>100	>100	>100	>100	75	78	67	99	87	57	102	82	88
18	5.6	2.9	4.7	5.6	3.3	4.5	>100	>100	>100	22	35	26	30	32	23	101	124	96
19	>100	>100	>100	>100	>100	>100	>100	>100	>100	83	97	98	99	103	100	98	99	92
20	>100	>100	>100	>100	>100	>100	>100	>100	>100	106	113	118	93	87	86	91	100	78
21	9.8	5.6	7.3	5.4	3.5	4.1	>100	>100	>100	27	16	7	4	2	0	87	95	105
22	4.2	2.8	2.1	3.4	1.9	1.5	>100	>100	>100	38	53	46	38	37	23	108	100	103
23	3	2.6	2.7	1.6	1.4	1.8	>100	>100	>100	43	42	18	31	22	8	94	120	85
24	>200	>200	>200	77	90	110	35	24	53	82	74	78	34	43	53	26	19	20
25	40*	43*	39*	>200	>200	>200	>200	>200	>200	191	194	187	90	83	70	98	79	95
26	32*	35*	35*	>200	>200	>200	>200	>200	>200	207	232	226	95	100	83	103	97	85
27	56*	46*	41*	210	140	147	220	140	130	167	160	155	52	45	42	53	41	40
28	>200*	>200*	>200*	>200	>200	>200	>200	>200	>200	123	126	111	85	82	81	101	94	91
29	8*	8*	16*	>200	>200	>200	>200	>200	>200	174	163	195	101	97	71	99	76	93
E_2	>100	>100	>100	>100	>100	>100	>100	>100	>100	86	88	77	97	90	89	99	95	99
Tam *E*	45	95	69	4.5	5.4	6	>100	>100	>100	37	46	36	30	9	30	102	112	93
Tam *Z*	77	92	59	5.5	4.4	3.2	>100	>100	>100	45	47	49	31	15	21	111	116	107
OHTam *E*	33	59	20	5.9	4.2	5.7	>100	>100	>100	30	30	32	17	0	14	107	104	100
OHTam *Z*	29	56	33	7.3	4.9	4.5	>100	>100	>100	20	22	27	10	13	2	104	123	94

Stimulation and inhibition of PKC subspecies activity by TPEs and DPEs were determined using either H1 histone as substrate with 0.1 mM Ca^{2+} and 2 μg/ml phosphatidylserine in the absence (PS) or presence (PS + DO) of 0.2 μg/ml 1,2-diolein, or using protamine sulfate as substrate with 0.5 mM

(χ^2 distance) and the Prim algorithm was applied [34, 35]. This method links the test-compounds within a network in which no back-tracking is permitted. The distance separating two test-compounds, as measured along the pathways generated, is an indication of the closeness of the relationship between them with respect to all the biochemical parameters measured. It takes into account both specificity and amplitude.

Calculations were performed on a microcomputer (Hewlett-Packard 9836) with a program adapted for BASIC from FORTRAN Anacor software. The factorial map was drawn directly on a digital plotter with a precision of 1/100 in. (but has been redrawn for the purposes of this paper). A simplified version of the basic CFA program for running on an IBM PC compatible computer is available upon request from J.-C. Doré (Muséum National d'Histoire Naturelle, 63 rue Buffon, 75005 Paris, France).

RESULTS

Effect of DPEs and TPEs on PKC activity: examination of the data prior to multivariate analysis

We determined the effect of TPEs and DPEs on types I (γ), II (β) and III (α) PKC subspecies purified from rat brain cytosol under three different conditions of enzyme activation that, for simplification will be denoted in the text, as follows:

(a) "with low PS": PKC activity was determined in the presence of Ca^{2+} and a low PS concentration (2 μg/mL) which gives partial activity; both activation and inhibition can be detected under these conditions [18–20].

(b) "with PS + DO": PKC activity was determined as above (Ca^{2+} and PS) but in the presence of diacylglycerol (0.2 μg/mL DO) which increases activity about 5-fold [18]. These conditions identify compounds able to inhibit PKC activation by the physiological activator, diacylglycerol.

(c) "with protamine sulfate": When protamine sulfate is used as a phosphate acceptor, PKC exhibits full activity in the absence of phospholipid, diacylglycerol and Ca^{2+} [2]. Inhibition of this enzyme activity is therefore independent of the presence of the lipid cofactors.

The results are summarized in Table 2 and are expressed as effective (EC_{50}) or inhibitory (IC_{50}) concentrations and as a percentage of the activity of the control. Insofar as it is possible to interpret such voluminous data "de visu", it would seem that the results for types I (γ), II (β) and III (α) PKC are similar but that modulation of PKC activity is a function of the mode of enzyme activation and of the chemical structure of the TPEs and DPEs. All TPEs with at least one basic dialkylaminoethoxy group inhibited PKC activated "with low PS" or "with PS + DO" at a relatively low concentration (IC_{50} of 1–10 μM). "With PS + DO", tamoxifen derivatives inhibited PKC in the same concentration range, but "with low PS", 10-fold higher concentrations were required. Increasing PS concentration overcame the inhibitory effect of the basic amino-substituted TPEs according to sigmoidal dose–response curves (data not shown) which may reflect a PS–TPE interaction [19, 20]. These TPEs did not compete with DO (data not shown) nor did they inhibit protamine sulfate phosphorylation (Table 2). Their mechanism of action is probably similar to that of the two lead compounds (TPEs **13*E*** and **14**) which were shown to interact with the phospholipid cofactor and the regulatory domain of the enzyme [19, 20].

The other effectors of PKC, active at high concentrations (30–200 μM), are all hydroxylated on both the α and α' phenyl rings. Two subclasses can be distinguished; inhibitors in the TPE series and dual-type inhibitors/activators in the DPE series. TPEs **4**, **6**, **8** and **24** inhibited PKC activated "with PS + DO" or "with protamine sulfate" (Table 2) and also the activity of the catalytic subunit PKM in the same concentration range (IC_{50}s of 110, 78, 80 and 120 μM, respectively), suggesting that they interact with this domain. DPEs **25**, **26**, **27** and **29** with a 1,1-bis-(*p*-hydroxyphenyl) ethylene moiety stimulated PKC activated "with low PS" but inhibited PKC activated "with PS + DO", i.e. when enzyme activity increased 5-fold. DPE **27** was the most inhibitory "with PS + DO" and also "with protamine sulfate" (Table 2). We have previously shown [18] that DPEs **25–27** inhibit PKM with IC_{50}s of 250, 155 and 140 μM, respectively, suggesting that they interact with the catalytic domain. Unexpectedly, TPEs **11*Z*/*E*** also stimulated PKC activity "with low PS" but the mechanism remains to be clarified. The other TPEs were inactive whatever the mode of PKC activation.

This study of the TPE and DPE concentrations at which half maximal effects were recorded (EC_{50} and IC_{50}) was completed by multivariate analyses of the maximal effects observed.

Correspondence factorial analysis.

The $\phi_1\phi_2$ factorial map (Fig. 1) represents 87.6% of the total variance of the system and shows the position of the nine vectors, describing the influence of the TPE/DPE population on all the tested biochemical activities. The negative extremities of the vectors [e.g. PS(−)] correspond to specific and highly inhibitory responses, whereas their positive extremities [PS(+)] correspond to specific and highly stimulatory responses. The nine vectors form three groups reflecting the three different activation conditions of PKC. The proximity of the three vectors representing the different PKC subspecies (types I, II and III) inside each group confirms that the action of the TPE/DPEs on the different PKC isotypes is very similar. The further proximity of two groups of vectors along the ϕ_1 axis, moreover, suggests that there are analogies in the modulation of PKC activated "with low PS" and "with PS + DO", whereas the orthogonal position of the three vectors near the ϕ_2 axis that represents inhibition of PKC catalysed protamine sulfate phosphorylation suggests that this activity is unrelated.

The potential of a test-compound for a given biological activity can be deduced from its position within the map. Four main sub-populations of molecules can be distinguished and, for each sub-population, there are two or three levels of correlation with regard to their biological behavior. These are indicated by shading derived from a

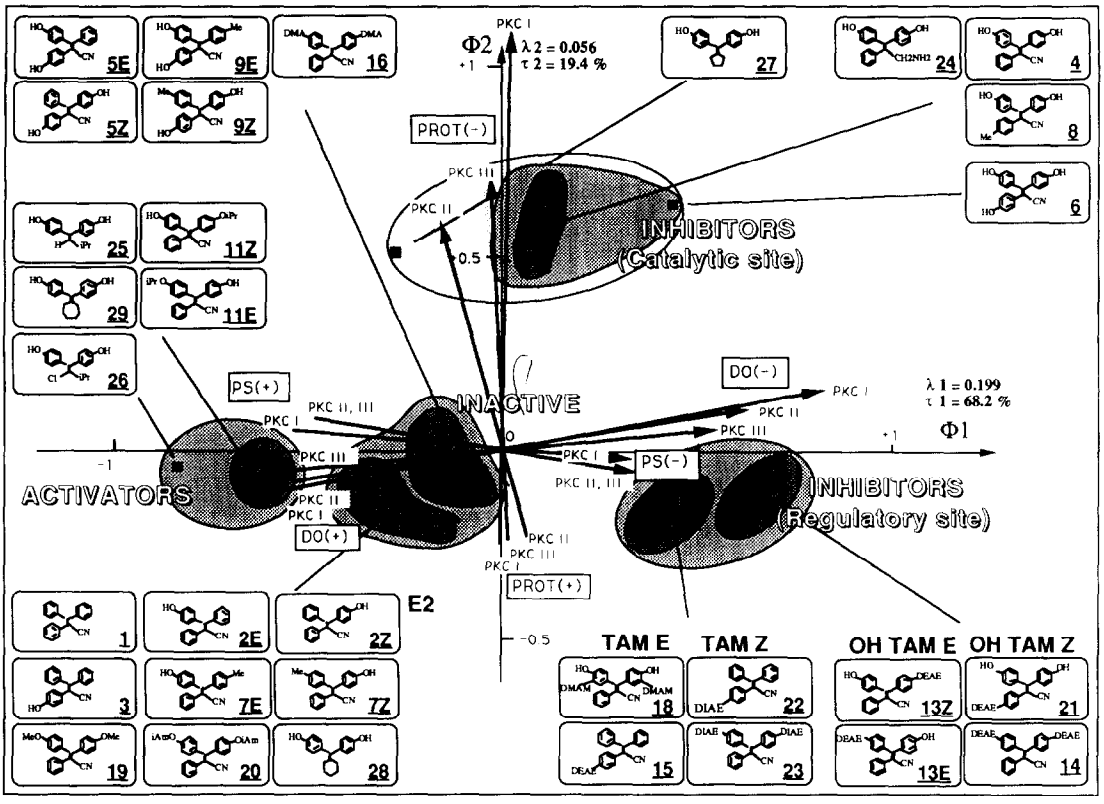


Fig. 1. $\Phi_1\Phi_2$ Factorial map obtained by multivariate analysis of the per cent data of Table 2. Each vector reflects the influence of the TPE/DPE population on a given PKC isotype under one particular activation condition. The negative extremities of the vectors [e.g. PS(−)] correspond to specific and highly inhibitory responses (for each activation condition), whereas the positive extremities PS(+) correspond to specific and highly stimulatory responses. The positions of the test-compounds with respect to these vectors reflect the specificity (proximity to a vector) and amplitude (the distance from the origin) of their effects. The shading, drawn according to a hierarchical ascending classification (not shown), represents different levels of correlation between the behavior of the test-compounds; the darker the shading, the more similar the molecules.

hierarchical ascending classification (not shown); the more intense the shading, the closer the behavior of the molecules. The sub-population near the "PS(−)" and "DO(−)" extremities of the vectors is constituted of TPEs substituted with a basic amino side-chain(s). The further they are from the origin, the more specific and inhibitory they are with respect to PKC activation by Ca^{2+} and PS plus or minus DO. The sub-population at the "PS(+)" extremity [DPEs with a 1,1-bis-(*p*-hydroxyphenyl) ethylene moiety and TPEs with an isopropyloxy substitution] is characterized by its ability to stimulate PKC activated "with low PS". Situated in an orthogonal position near the protamine sulfate "PROT(−)" extremity is a third sub-population of TPEs with a hydroxy group on both the α and α' phenyl rings, that is able to inhibit protamine sulfate phosphorylation by PKC and also to exert other actions on PKC, as indicated by the dispersion of the molecules around this pole [e.g. slightly atypical behavior of TPE **6**, which inhibited PKC whatever the activation conditions of the enzyme, and of DPE **27** (see above)]. The sub-population near the origin is composed of all the molecules (estradiol, non-amino-substituted TPEs, TPEs mono- and di-hydroxylated on their α and β or α' and β phenyl rings) that are inactive on all PKC subspecies whatever the mode of activation.

The CFA described above highlighted the relations between chemical structures and mechanisms of PKC modulation. The detailed structure of the molecules, their specificity and efficacy in modulating PKC are discussed below.

Minimum spanning tree

In the minimum spanning tree (Fig. 2) describing the data of Table 2, the molecules located at the top of the tree are the most, and those at the bottom are the least specific and potent. The gradient between these extremes enables an analysis of the influence of chemical substituents on specificity and activity.

The root of the tree starts with the unsubstituted TPE **1** which has no activity towards any PKC subspecies, whatever the mode of activation. The trunk of the tree is constituted of inactive TPEs. These are mono-hydroxylated on one of their phenyl rings (TPEs **2Z**/***E***, **3**, **7Z**/***E***) and/or substituted by a small hydrophobic substituent (**7Z**/***E***, **19**). The tree then spreads out into three well-defined branches.

The top left-hand branch (group A) includes all

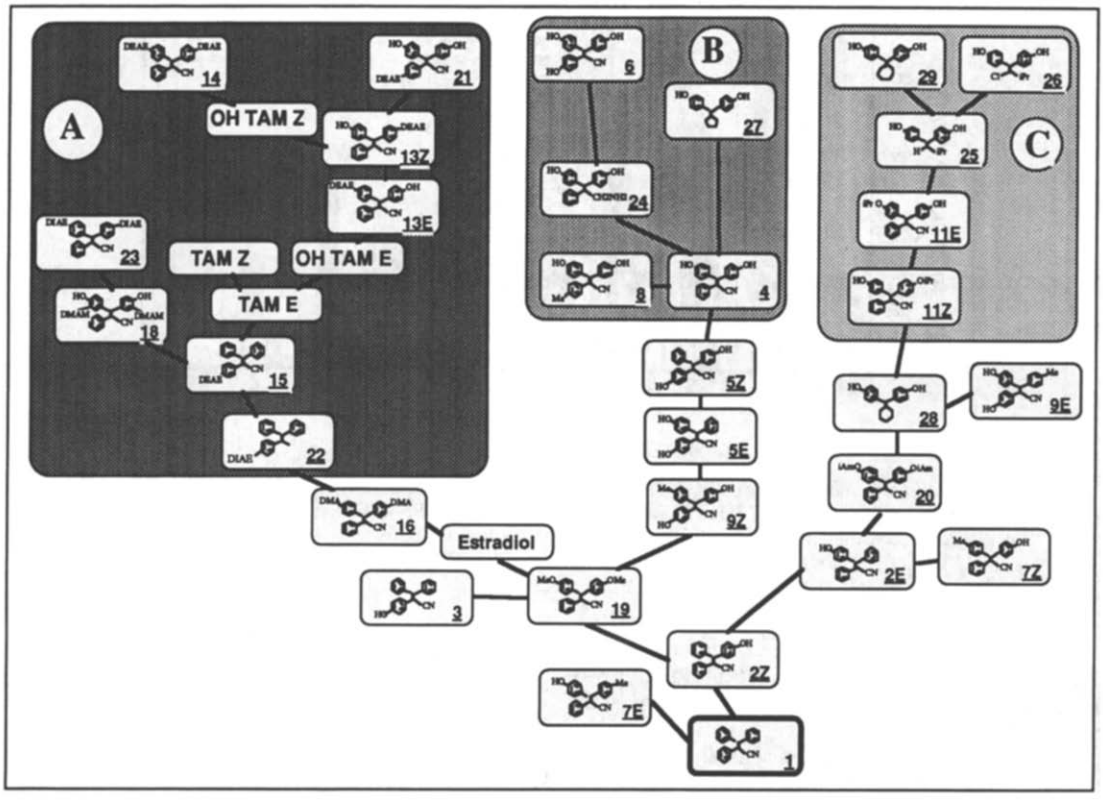


Fig. 2. Minimum spanning tree obtained from the data in Table 2. The molecules are distributed along various branches as a function of their properties with regard to PKC. The molecules located at the top of the tree are the most specific and potent, those at the bottom, the least. The distance between two molecules reflects analogy in their ability to modulate PKC. Groups A, B and C represent the test-compounds able to inhibit PKC activated by Ca^{2+} and PS plus or minus DO, to inhibit the phosphorylation of protamine sulfate by PKC, and to stimulate PKC activity in the presence of Ca^{2+} and PS, respectively.

TPEs substituted with a basic amino side-chain(s). These TPEs inhibit the activation of PKC by Ca^{2+} and PS plus or minus DO, and are presumed to interact with the regulatory domain of the enzyme and with phospholipid. Three main structural features characterize this group. Firstly, the presence of a sufficiently long basic amino side-chain is required since TPE **16** (just below group A) substituted on the α and α' phenyl rings with a dimethylamino group is inactive, whereas TPEs **14** and **23** with longer dialkylamino side-chains are much more active; secondly, substitution by two dialkylamino side-chains is favorable (see the extreme positions of TPEs **14** and **23**); thirdly, hydroxylation of the phenyl ring seems to increase activity (compare the locations of TPEs **15** and **21**, Tam *E* and OHTam *E*, Tam *Z* and OHTam *Z*).

The top right-hand branch (group C) is composed of DPEs **25**, **26**, **29** with a 1,1-*p*(hydroxyphenyl) moiety and TPEs **11Z**/*E* with an isopropyloxy group which stimulate PKC activity in the presence of Ca^{2+} and PS. The most active and specific are DPEs hydroxylated on their α and α' phenyl rings (DPEs **25**, **26** and **29**) which probably interact with the catalytic domain of the enzyme. DPE **28**, also with a 1,1-*p*-(hydroxyphenyl) moiety but located lower down the branch, is inactive.

The middle branch (group B) is composed mainly of TPEs with an α and α' hydroxy group which are characterized by their ability to inhibit the phosphorylation of protamine sulfate by PKC but which can also have other actions on PKC: TPEs **4**, **6**, **8** and **24** inhibited PKC activity in the presence of Ca^{2+}, PS and DO as well as the activity of the catalytic fragment of the enzyme, suggesting that they interact with the catalytic domain. The extreme positions of TPE **6** and DPE **27** in two different sub-branches probably reflect their specificity to modulate (inhibition and activation, respectively) PKC activity in the presence of Ca^{2+} and PS. Only compounds di-hydroxylated on the α and α' phenyl rings belong to this group. TPEs di-hydroxylated on β and α or α' phenyl rings are inactive but their proximity indicates that they may have a somewhat higher specificity and activity than other more distant molecules.

It is worth noting that the addition of a basic diethylaminoethoxy side-chain on the β phenyl ring of TPE **4** (di-hydroxylated on both the α and α' phenyl rings) to give TPE **21** suppressed the inhibition of protamine sulfate phosphorylation by PKC. A similar effect was observed on addition of a basic dimethylaminomethyl group at the meta position of both the α and α' phenyl rings of TPE **4** to give TPE

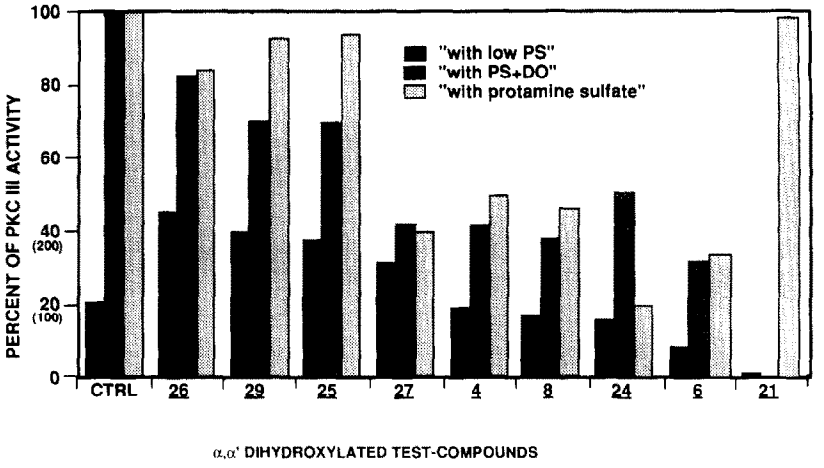


Fig. 3. Relative activities of PKC III in the presence of α,α'-di-hydroxylated test-compounds. Experimental values for PKC III activated with low PS and with PS + DO are expressed as percentages of the control values obtained with PS + DO (or with low PS between parentheses). The activity of PKC III activated with protamine sulfate is expressed as the percentage of its own control value.

18. The presence of at least one basic amino side-chain, therefore, decreases efficient interaction with the catalytic domain of the enzyme, whereas the presence of a 1,1-bis-(*p*-hydroxyphenyl) moiety does not seem to modify interaction with the regulatory domain of the enzyme or with phospholipid.

DISCUSSION

The present study has explored by multivariate analysis the structural determinants governing the mechanism of PKC modulation by DPEs and TPEs. Two complementary methods (i.e. CFA and minimum spanning tree) have illustrated clearly the significance of the results obtained by screening 37 molecules on 18 parameters (Table 2) and have revealed that molecules sharing a common structural feature inhibit or activate PKC by the same route.

The basic amino-substituted TPEs inhibited PKC activation at relatively low concentrations (IC_{50} of 1–10 μM), probably by interaction with the phospholipid cofactor. Although saturating concentrations of PS-vesicles can overcome this inhibition, there is no evidence for competition between TPE and phospholipid for the enzyme. It is plausible that these TPEs, by altering the phospholipid lamellar structure, inhibited the interaction of PS with the enzyme and/or with its substrates [36, 37]. This type of inhibition was obtained without exception for all TPEs substituted with at least one basic dialkylaminoethoxy side-chain, although to slightly different extents depending upon the position of the substitution(s). The low specificity suggests that these molecules, probably on account of their common polyaromatic hydrophobic moiety and their charged amino side-chain(s), may act like tranquilizers [38], local anesthetics [38], Adriamycin [39], trifluoroperazine [39] or rhodamine derivatives [40]. However, compared to these synthetic polyaromatic structures, the TPE skeleton generally leads to higher efficacy.

The basic amino-substituted TPEs may also interact directly with the regulatory domain of PKC, as we have shown previously for TPEs **13*E*** and **14** [19, 20], but their binding site on this domain remains undefined since they were not competitive with respect to diacylglycerol, Ca^{2+} and ATP [19, 20], and, unlike basic amino acridine derivatives [41], they did not inhibit phorbol ester binding [E. Bignon, unpublished results]. Interaction with the regulatory domain has been described for the microbial compound Calphostin C (UCN 1028 C) and related structures that are potent specific PKC inhibitors (IC_{50} of 0.05 μM) [42]. These inhibitors lacking the basic amino side-chain probably interact with the phorbol ester binding site and, unlike other polyaromatic structures and TPEs, do not appear to interact with the phospholipid cofactor.

The other effectors of PKC activity in our series possess a 1,1-bis-(*p*-hydroxyphenyl) ethylene moiety and influenced PKC activity at high concentrations (30–200 μM), probably by interacting with the catalytic domain of the enzyme. As revealed in Fig. 3 for PKC III, the experimental values for the intensity of the inhibitory or stimulatory actions of these $\alpha\alpha'$-di-hydroxylated derivatives suggest that two phenomena might be involved in their modulation of PKC activity: on the one hand, there was a low but systematic enhancement of the catalytic activity measured "with low PS" with respect to that measured "with PS + DO". The low PS activity, which was 20% of PS + DO activity for the control, rose to 35–50% of this activity in the presence of these derivatives. This effect was, in all likelihood, due to their common $\alpha\alpha'$-di-hydroxylated diphenylthylene structural core. On the other hand, an overall decrease of PKC activity was noted that could be explained by the other substituents on the central double bond, a β-phenyl ring (substituted or not) being the most inhibitory of these. This decrease occurred under all three conditions of enzyme activation and, as evidenced by the parallelism between results obtained "with PS + DO" and "with

protamine sulfate", it most probably also resulted from action at the catalytic site. If this were the case, the dual activator/inhibitor effect observed with these TPEs and DPEs would be the result of a balance between two opposing actions at the same domain site. However, TPE **6** and DPE **27** respectively inhibited and stimulated PKC "with low PS" slightly more than expected, as also revealed by their deviant position in Fig. 1. TPE **21** behaved very differently on account of the basic side-chain on its β-phenyl ring which is responsible for strong inhibition of the regulatory site of the enzyme.

In the naphthalenesulfonamide series, the substitution of a phenyl ring on the hydrocarbon chain of the inhibitor W7 also profoundly changes behavior, since the resulting molecule SC9 is an activator [43]. We have shown that the trihydroxylated TPE **6** inhibits PKC in a competitive manner with respect to ATP [20]. Similar behavior has been reported for flavonoid derivatives [44, 45] and the tyrosine kinase inhibitor erbstatin [46], suggesting that several hydroxy groups on a specific aromatic structure might be important for interaction with the catalytic domain. It is, however, difficult to find a common structural feature of other PKC inhibitors supposed to interact with the catalytic domain of the enzyme, such as polysulfonated naphthylurea suramin [47], indolocarbazoles [staurospaurine and its derivatives (e.g. K252a and CGP41 251)] [48–51], naphthalene-(W7) and isoquinoline-sulfonamides (H7, H8) [52–54]. Whether this diversity could be due to the presence of distinct sites of interaction on the catalytic domain is not known.

The above relationships between structure and mechanism should be of use in the design of more potent and specific inhibitors of PKC. They have, for instance, highlighted the disadvantage of basic amino-substituted polyaromatic structures that are liable to interact non-specifically with the phospholipid cofactor and emphasized the possibility of different interactions with the catalytic domain of PKC. Although this study did not determine the specificity of all the molecules with respect to other kinases, we have not observed any effect on the catalytic fragment of protein kinase A for the three lead TPEs (**13*E***, **14** and **6**) [19]. TPEs such as tamoxifen can, however, inhibit other kinases, e.g. Ca^{2+}/calmodulin-dependent protein kinase [55, 56]. Moreover, this study has pointed out the difficulty in developing inhibitors specific to one type of PKC, probably because the enzymatic properties of the α, β or γ subspecies differ only slightly. This may not be the case for the recently discovered family of type δ, ε and ζ PKC subspecies which lack the C2 conserved region in the regulatory domain and whose kinase activity is clearly independent of the presence of Ca^{2+} [1].

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