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Characterization of phorbol esters activity on individual mammalian protein kinase C isoforms, using the yeast phenotypic assay

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Abstract

An alternative in vivo assay, based on growth inhibition of yeast expressing an individual mammalian protein kinase C (PKC) isoform (proportional to the degree of PKC activation), was used to characterize the activities of phorbol-12-myristate-13-acetate (PMA) and its analogues on classical (α and βI), novel (δ and η) and atypical (ζ) PKC isoforms. Effects of PMA, 4α-PMA, phorbol-12-myristate-13-acetate-4-*O*-methyl-ether (MPMA), phorbol-12-monomyristate (PMM), phorbol-12,13-diacetate (PDA), phorbol-13-monoacetate (PA), phorbol-12,13-dibutyrate (PDB), phorbol-12,13-didecanoate (PDD) and 12-deoxyphorbol-13-phenylacetate-20-acetate (dPPA), on growth of yeast expressing individual PKC isoforms was determined. PMA-induced growth inhibition on all isoforms tested (except on PKC-ζ). PDD and PDB presented an efficacy similar to PMA; the other PMA-analogues presented lower efficacies. MPMA and 4α-PMA stimulated growth of yeast expressing classical PKCs and reduced the PMA-induced growth inhibition, effects similar to those exhibited by the PKC inhibitors chelerythrine and R-2,6-diamino-*N*-[[1-(1-oxotridecyl)-2-piperidinyl]methyl]-hexanamide dihydrochloride (NPC 15437). This study reveals that phorbol esters differ on their potency to activate a given PKC isoform, and presents their isoform-selectivity. Furthermore, MPMA and 4α-PMA caused effects similar to those expected from PKC inhibition.

Keywords: Protein kinase C; Isoform; Phorbol ester; Yeast phenotypic assay

1. Introduction

The ubiquitous serine–threonine kinase, protein kinase C (PKC), is a family of several isoforms grouped according to their primary structure and cofactor requirements: the classical PKCs, which include the isoforms α, βI, βII and γ, require phospholipid, diacylglycerol and Ca^{2+} for activation; the novel PKCs, which include the isoforms δ, ε, η and θ, require phospholipid, diacylglycerol but not Ca^{2+}; the atypical PKCs, which include the isoforms ζ and λ/ι, depend on phospholipid but not on diacylglycerol or Ca^{2+} (Musashi et al., 2000). More recently, a fourth group of structurally distinct PKCs has been identified, the so-called PKC-related kinases (PRKs; Webb et al., 2000).

PKC actively participates in a diversity of signaling pathways that control cell proliferation, differentiation, survival, transformation and apoptosis (Musashi et al., 2000; Webb et al., 2000; Yang and Kazanietz, 2003). However, the elucidation of the precise role of each isoform has been hampered by the lack of isoform-selective PKC ligands (Hofmann, 1997; Way et al., 2000; Yang and Kazanietz, 2003).

Phorbol esters have been, for many years, the preferred pharmacological activators to study PKC (Barry and Kazanietz, 2001; Kazanietz et al., 2000; Yang and Kazanietz, 2003). Although 'non-PKC' receptors have being identified for phorbol esters, PKC isoforms still remain considered the main receptor for these compounds and, at present, phorbol esters are the most used pharmacological tools to elicit PKC activation. Therefore, the knowledge of phorbol esters activities and PKC isoform-selectivities is still an issue requiring further studies, particularly on systems where PKC-mediated effects are clearly identified.

Phorbol esters bind to PKC at the C1 domain of the PKC regulatory region that consists of a tandem repeat of cysteine-rich motifs, which is not found in atypical PKCs (Burns and Bell, 1991). Several in vitro assays have revealed that phorbol

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esters may differ on their potency to activate classical and novel PKC isoforms (Dimitrijevic et al., 1995; Evans et al., 1991; Ryves et al., 1991). Nevertheless, characterization of their isoform-selectivity in an in vivo system has been hampered by methodological limitations, such as the existence, in mammalian cells, of several PKC isoforms (see, for instance, Darbon et al., 1986; Huwiler et al., 1994; Iannazzo et al., 1999; Shoyab and Todaro, 1980) and of phorbol ester receptors other than PKC (Kazanietz et al., 2000; Ohno and Nishizuka, 2002; Yang and Kazanietz, 2003). The in vitro assays tried to overcome these limitations (Geiges et al., 1997; Ryves et al., 1991), but their cost, the impossibility to study the isoform in a cellular environment, and to reproduce the interactions between PKC and other cellular constituents has been considered a limitation for the use of such assays (Webb et al., 2000).

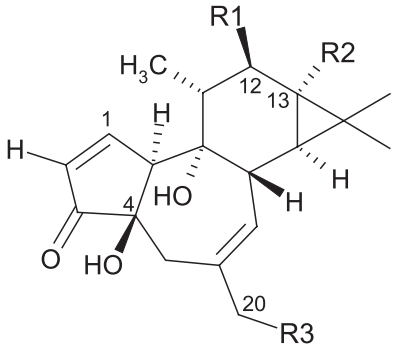
The yeast phenotypic assay is an alternative in vivo assay that allows comparison of effects of phorbol esters on individual mammalian PKC isoforms, due to the nonresponsive putative phorbol esters receptors in yeast cells (Riedel et al., 1993a,b; Shieh et al., 1995, 1996). On this assay, PKC activators cause an inhibition of growth of yeast that express a mammalian PKC isoform (reflecting an increase in the cell doubling time), which is proportional to the degree of PKC activation (Riedel et al., 1993a,b; Shieh et al., 1995, 1996); PKC inhibitors reduce that growth inhibition and may cause, per se, stimulation of yeast growth (Keenan et al., 1997).

In the present study, effects of nine phorbol esters (Fig. 1) on isoforms α and βI of the classical PKC family, δ and η of the novel PKC family and ζ of the atypical PKC family were compared using the yeast phenotypic assay. The phorbol esters tested differ on the substituents at C-4, C-12, C-13 or at C-20 of the phorbol skeleton, positions previously considered important for the activity of these compounds as PKC activators. Our results show that phorbol esters differ on their efficacy and potency towards the PKC isoforms assayed, and further indicate that some may cause effects similar to those elicited by PKC inhibitors.

2. Materials and methods

2.1. Reagents

The kit for protein quantification was from Pierce (Biocontec, Lisboa, Portugal). The secondary alkaline phosphatase-conjugated anti-rabbit IgG detection kit (AP-10), recombinant proteins PKC-α (PK11), PKC-βI (PK16), PKC-δ (PK31), PKC-η (PK46) and PKC-ζ (PK41) were from Oxford Biomedical Research (LabClinics, Barcelona, Spain). Nitrocellulose membranes and all the reagents for sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and immunoblots were from BioRad (PACI, Lisboa, Portugal). Yeast nitrogen base was from DIFCO (Merck Portugal, Lisboa, Portugal). Acid-washed glass beads, antibodies to PKC-α, PKC-βI, PKC-δ, PKC-η and PKC-ζ, aprotinin, arachidonic acid sodium salt, chelerythrine chloride, leupeptin, R-2,6-diamino-*N*-[[1-(1-oxotridecyl)-2-piperidinyl]methyl]-hexanamide dihydrochloride (NPC 15437 dihydrochloride), pepstatin A, phenylmethylsulfonyl fluoride, phorbol 12,13-diacetate (PDA), phorbol 12,13-dibutyrate (PDB), phorbol 12,13-didecanoate (PDD), phorbol 12-monomyristate (PMM), phorbol 12-myristate 13-acetate (PMA), phorbol 12-myristate 13-acetate 4-*O*-methyl



	R1	R2	R3
Phorbol 12-myristate 13-acetate (PMA)	-OCO$(CH_2)_{12}CH_3$	-$OCOCH_3$	-OH
4α-Phorbol 12-myristate 13-acetate (4α-PMA)[a]	-OCO$(CH_2)_{12}CH_3$	-$OCOCH_3$	-OH
Phorbol 12-myristate 13-acetate 4-O-methyl ether (MPMA)[b]	-OCO$(CH_2)_{12}CH_3$	-$OCOCH_3$	-OH
Phorbol 12,13-didecanoate (PDD)	-OCO$(CH_2)_8CH_3$	-OCO$(CH_2)_8CH_3$	-OH
Phorbol 12,13-dibutyrate (PDBu)	-OCO$(CH_2)_2CH_3$	-OCO$(CH_2)_2CH_3$	-OH
Phorbol 13-monoacetate (PA)	-OH	-$OCOCH_3$	-OH
Phorbol 12,13-diacetate (PDA)	-$OCOCH_3$	-$OCOCH_3$	-OH
Phorbol 12-monomyristate (PMM)	-OCO$(CH_2)_{12}CH_3$	-OH	-OH
12-Deoxyphorbol 13-phenylacetate 20-acetate (dPPA)	-H	-OCO$CH_2C_6H_5$	-$OCOCH_3$

Fig. 1. Chemical structures of the phorbol esters tested; [a]4α-PMA is a stereoisomer of PMA having an α-oriented hydroxyl group at C-4; [b]MPMA differs from PMA by having a methoxyl group at C-4.

ether (MPMA), phorbol 13-monoacetate (PA), 4α-phorbol 12-myristate 13-acetate (4α-PM*A*) were from SIGMA (Sigma Aldrich Química, Sintra, Portugal). 12-Deoxyphorbol 13-phenylacetate 20-acetate (dPPA) was from Biomol (Quimigranel, Madrid, Spain). All other chemicals used were of analytical grade.

2.2. Yeast transformation and cell cultures

Constructed yeast expression plasmids YEp52 and YEp51, encoding cDNA for bovine PKC-α and for rat PKC-βI, respectively (kindly offered by Dr. Heimo Riedel, Wayne University, Detroit, USA) and YEplac181, encoding the cDNA for the rat PKC-δ, mouse PKC-η or PKC-ζ (kindly offered by Dr. Nigel Goode, Royal Veterinary College, London, UK) were amplified in *Escherichia coli* (*E. coli*) DH5α and confirmed by restriction analysis. The plasmids used contain galactose-inducible transcriptional elements and the *leu2* gene for selection. *Saccharomyces cerevisiae* (*S. cerevisiae*; strain CG379; α *ade5 his7-2 leu2-112 trp1-289*α *ura3-52 [Kil-O]*; Yeast Genetic Stock Center, University of California, Berkeley, USA) was transformed using the lithium acetate method (Ito et al., 1983). To ensure selection of transformed yeast, cells were grown in leucine-free medium, in 1.5% agar plates, at 30 °C.

For the yeast phenotypic assay, transformed cells were incubated at 30°C in leucine free-medium, with slow shaking. The leucine free-medium contained 0.7% yeast nitrogen base, 2% glucose (w/v) or the indicated carbon source, amino acids, purines and pyrimidines, according to the transformed yeast requirements. Galactose (2%; w/v), instead of glucose, was included to induce transcription of the mammalian PKC gene.

2.3. Cell lysis and immunoblotting

Cell lysis was performed basically as described (Riedel et al., 1993a). The protein concentration was determined using a kit for protein quantification (*Coomassie Protein Assay Reagent Kit*, Pierce, Biocontec). Similar amounts of protein (~ 40 μg) from protein extracts were then separated on 10% SDS-polyacrylamide gels (Mini-Protean II, BioRad, Hercules, CA, USA). Positive controls (4 μg) were obtained using recombinant proteins PKC-α (MW 76,799 Da), PKC-βI (MW 76,790 Da), PKC-δ (MW 77,517 Da), PKC-η (MW 77,600 Da) and PKC-ζ (MW 67,740 Da). Proteins were electrophoretically transferred to nitrocellulose membranes and probed on immunoblots with specific rabbit antibodies to the individual mammalian PKC isoforms and revealed with a secondary alkaline phosphatase-conjugated anti-rabbit IgG (AP-10, Oxford Biomedical Research, LabClinics).

2.4. Yeast phenotypic assay

Transformed yeast cultures were routinely incubated in leucine-free glucose medium. Optical density values, measured at 620 nm (OD_{620}; Cary 1E Varian spectrophotometer, Palo Alto, CA, USA), were used as an indicator of growth. Transformed yeast were grown to an OD_{620} of approximately 1, collected by centrifugation and diluted to an OD_{620} of 0.05, in medium containing 2% (w/v) galactose (gene transcription-inducer) and 3% (v/v) glycerol (alternative carbon source). Diluted cultures (200 μl) were transferred to 96-wells microtitre plates and incubated for up to 100 h (150 h in experiments carried out to obtain growth curves), with slow shaking at 30 °C, either in the presence of drugs or solvent (0.1% dimethylsulfoxide; DMSO; final concentration). Growth was monitored by determining the OD_{620} using a plate reader (BioRad Benchmark Microplate Reader; Hercules, CA, USA). The difference between maximal OD_{620} reached and that measured at the beginning of incubation was used as an index of yeast growth. Drugs or solvent were added at the beginning and kept throughout the incubation. Yeast growth in the presence of drugs was expressed as percentage of growth observed in the presence of solvent; it was further transformed into growth inhibition by subtracting that value from 100. Therefore, 100% growth inhibition would occur when there was no growth during incubation; 0% growth inhibition would occur

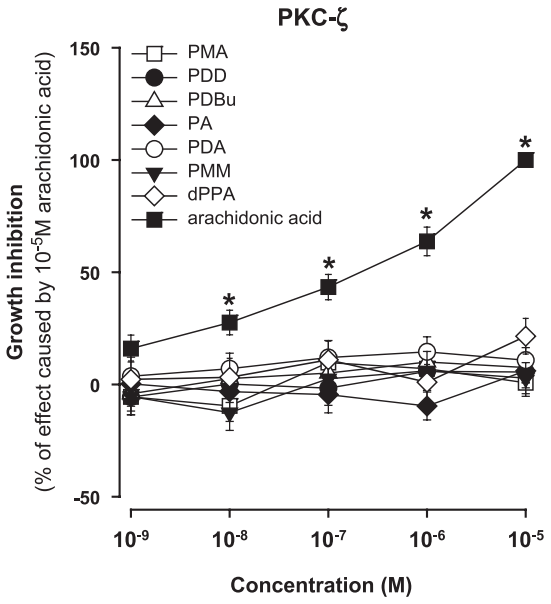


Fig. 2. Concentration–response curves for the effects of phorbol esters and arachidonic acid on growth of yeast expressing mammalian PKC-ζ. Transformed yeast were incubated with drugs or solvent (0.1% DMSO) and effects measured at 45-h incubation. Results are expressed as % of the maximal effect caused by 10^{-5} M arachidonic acid; 100% growth inhibition was considered that caused by 10^{-5} M arachidonic acid; 0% growth inhibition was considered to occur when growth in the presence of drugs was identical to that in the presence of solvent; negative growth inhibition was considered to occur when growth in the presence of drugs was higher than that in the presence of solvent and was taken to indicate growth stimulation. Shown are means ± S.E.M. of 16–36 determinations. Significant differences from control: $*P<0.05$ (paired Student's *t*-test).

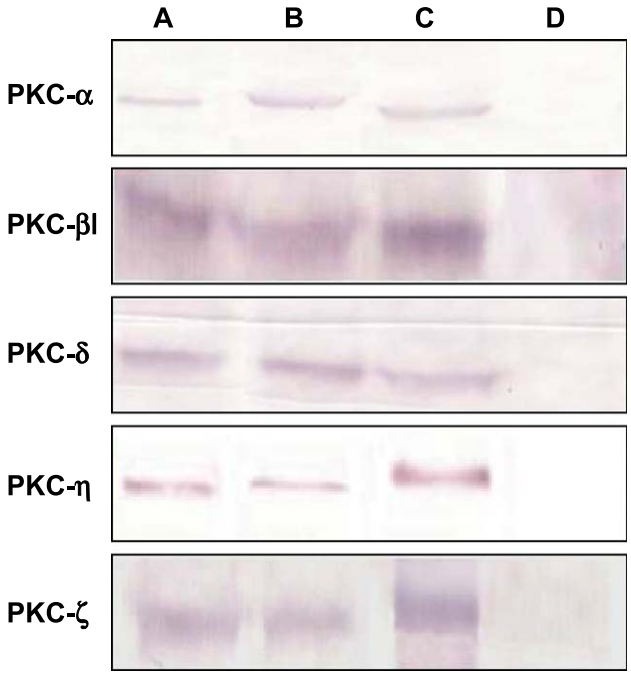


Fig. 3. Immunodetection of PKC-α, -βI, -δ, -η and -ζ isoforms expressed in transformed *S. cerevisiae* (CG379). Individual immunoblots are presented in a horizontal arrangement and were obtained from proteins extracts (~ 40 μg protein/lane) from cultures grown in selective medium with 2% galactose (lanes A and B; duplicate samples) or without 2% galactose (lane D). Positive controls (lane C; 4 μg) were obtained using recombinant proteins PKC-α (MW 76,799 Da), PKC-βI (MW 76,790 Da), PKC-δ (MW 77,517 Da), PKC-η (MW 77,600 Da) and PKC-ζ (MW 67,740 Da).

when growth in the presence of drugs was identical to that obtained in the presence of solvent; a negative growth inhibition occurred when growth in the presence of drugs was higher than in the presence of solvent and was taken as an indicator of drug-induced growth stimulation.

In experiments where drug potencies were compared, and because growth inhibition caused by a maximal concentration of phorbol esters varied between isoforms, 100% growth inhibition was standardized and assumed to be that caused by 10^{-5} M PMA on classical and novel PKC isoforms. Since atypical PKC isoforms are not activated by phorbol esters (Ways et al., 1992; see also Fig. 2), the activator used was arachidonic acid (up to 10^{-5} M), an unsaturated fatty acid known to activate PKC-ζ (Nakanishi and Exton, 1992; see also Fig. 2). Effects of phorbol esters were expressed as percentage of that growth inhibition.

Chemical structures of the phorbol esters used are depicted in Fig. 1. They differ from PMA on the substituents at C-12 and C-13, except 4α-PMA, which is a stereoisomer of PMA having an α-oriented hydroxyl group at C-4, and MPMA, which differs from PMA by having a methoxyl group at C-4. dPPA has also an acetyl group at C-20, and was included because it has been described as a PKC-βI selective activator (Evans et al., 1991; Ryves et al., 1991).

Table 1
Growth inhibition caused by 10^{-5} M of the compounds tested on transformed yeast cells incubated on glucose medium (absence of PKC expression)

Compounds	Growth inhibition (% of control)
Arachidonic acid	1.66 ± 1.4
PMA	1.79 ± 1.8
PDD	1.82 ± 0.9
PDB	1.22 ± 1.1
PA	− 0.86 ± 2.0
PDA	1.01 ± 1.8
PMM	− 0.94 ± 1.4
dPPA	1.00 ± 1.1
MPMA	0.72 ± 1.4
4α-PMA	1.12 ± 1.9

Growth in the presence of solvent was considered to be 0% growth inhibition (100% growth). Each value represents the mean ± S.E.M. of 20 determinations.

To obtain concentration–response curves, each phorbol ester was tested in concentrations ranging from 10^{-13} to 10^{-5} M and the concentration that caused 50% of the growth inhibition caused by 10^{-5} M PMA (arachidonic acid for PKC-ζ; EC_{50}) was calculated. For those isoforms in which phorbol esters caused less than 50% growth inhibition, determination of EC_{50} was not feasible and EC_{50} values are referred as nondeterminable (*ND*).

For interaction experiments, MPMA, 4α-PMA, chelerythrine and NPC 15437 were tested alone and combined with 10^{-5} M PMA at the same concentration (10^{-5} M; 10^{-6} M for NPC 15437), on yeast expressing PKC-α, -βI, -δ or -η. Results are expressed as % of growth inhibition.

2.5. Statistical analysis

Results are presented as arithmetic means ± S.E.M. and *n* represents the number of determinations. Differences between means were tested for significance using either paired Student's *t*-test, unpaired Student's *t*-test or one way

Table 2
Yeast growth inhibition caused by 10^{-5} M PMA on the PKC isoforms studied

PKC isoforms	Growth inhibition caused by 10^{-5} M PMA (% of control)
α	40.6 ± 1.9 (n = 36)
βI	36.1 ± 1.1 (n = 56)
δ	26.6 ± 0.6 (n = 52)
η	21.3 ± 0.7 (n = 52)
ζ	0.2 ± 1.4 (n = 36)[a]

Growth in the presence of solvent was considered to be 0% growth inhibition (100% growth). Each value represents the mean ± S.E.M. of the indicated *n* determinations.
[a] Growth inhibition caused by 10^{-5} M arachidonic acid was 23.9 ± 0.7 (n = 56).

analysis of variance (ANOVA), followed by Tukey's post hoc test.

A P value lower than 0.05 was considered to be statistically significant.

3. Results

3.1. Expression of mammalian PKC isoforms in S. cerevisiae

Plasmids containing the cDNA of PKC-α, -βI, -δ, -η or -ζ were amplified in *E. coli* DH5α and confirmed by restriction analysis. Plasmids were stably propagated in transformed *S. cerevisiae* (CG379) and selected in leucine-free medium. Transcription was routinely induced by 2% galactose.

To test whether PKC isoforms were faithfully expressed, protein extracts of transformed cells, grown in the presence or in the absence of the transcription inducer, were separated on (SDS)-polyacrylamide gels and analysed with specific antibodies in immunoblots. Expression of mammalian PKC isoforms was observed only in the presence of 2% galactose and resulted in a single antigenic band, which co-migrated with the respective recombinant protein (Fig. 3).

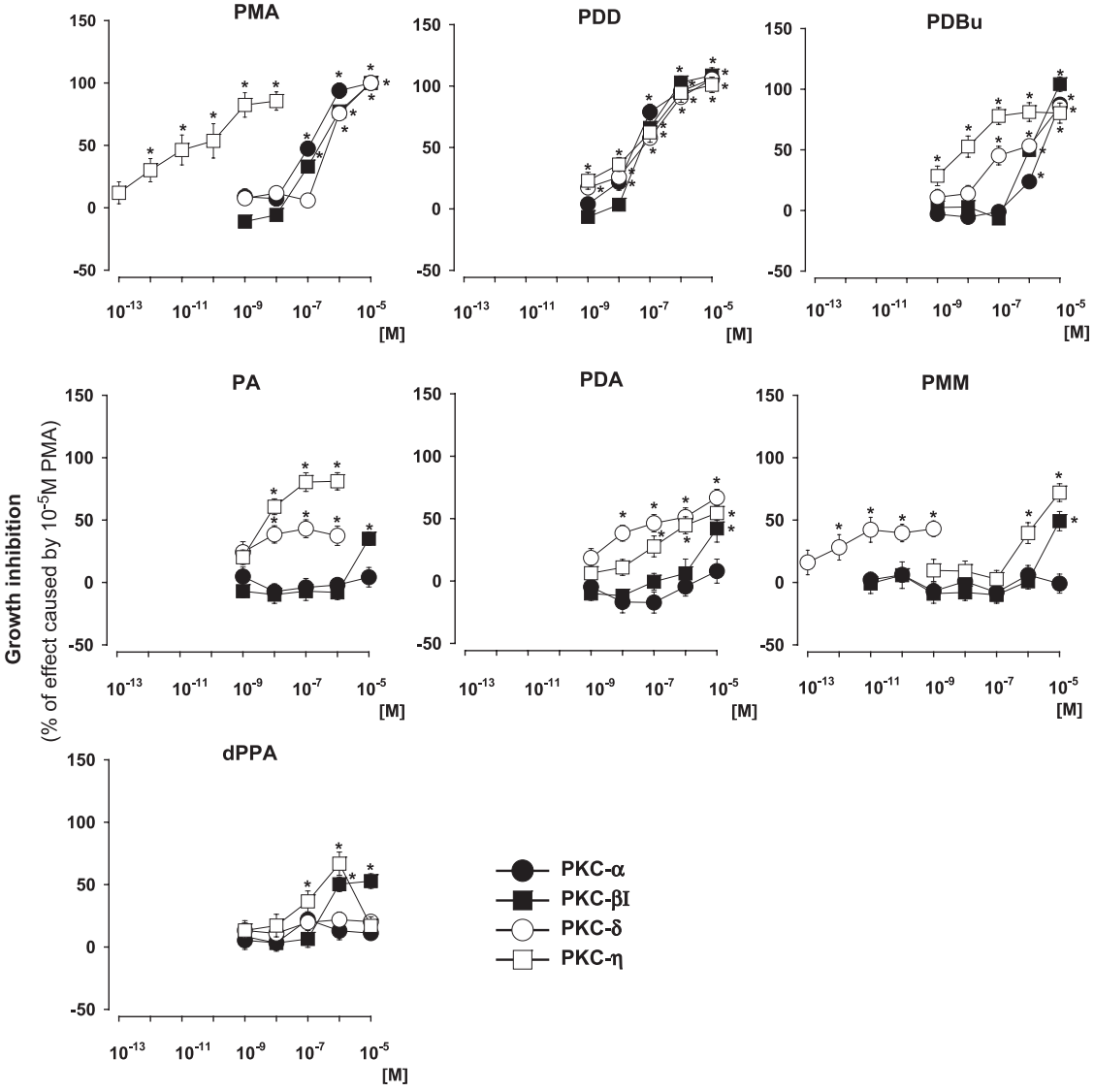


Fig. 4. Concentration–response curves for the effects of phorbol esters on growth of yeast expressing the indicated mammalian PKC isoforms (α, βI, δ or η). Transformed yeast cells were incubated with phorbol esters or solvent (0.1% DMSO) and effects measured at 65 h (for classical PKCs; α and βI) or at 45 h (for novel PKCs; δ and η) incubation. Results are expressed as % of the maximal effect caused by 10^{-5} M PMA; 100% growth inhibition was considered that caused by 10^{-5} M PMA; 0% growth inhibition was considered to occur when growth in the presence of drugs was identical to that in the presence of solvent; negative growth inhibition was considered to occur when growth in the presence of drugs was higher than that in the presence of solvent and was taken to indicate growth stimulation. Shown are means ± S.E.M. of 20–56 determinations. Yeast growth significantly different from solvent: $*P<0.05$ (paired Student's t-test).

3.2. Effects of phorbol esters in yeast growth

Two hundred microlitre cultures were incubated with or without expression of PKC isoforms (2% galactose present or absent, respectively). The solvent used (0.1% DMSO; final concentration) did not change yeast growth either in the presence or in the absence of 2% galactose.

Phorbol esters (as well as arachidonic acid) were tested in concentrations up to 10^{-5} M. Higher concentrations of phorbol esters were not possible to test due to their low solubility in the culture medium. In the absence of galactose, phorbol esters and arachidonic acid did not change yeast growth (Table 1).

In preliminary experiments, growth curves for yeast expressing PKC-α, -βI, -δ, or -η (2% galactose present) were obtained in the presence of solvent, of 10^{-5} M PMA (considered a standard activator for the classical and novel PKC isoforms) or of 10^{-5} M arachidonic acid (for yeast expressing PKC-ζ). In the obtained curves, logarithmic and stationary phases were identified for each isoform. Effects of drugs on yeast growth were monitored along the experiment to determine the period of time during which maximal growth inhibition was reached and remained fairly constant (''steady-state growth inhibition''). Estimation of drug effects was based on OD_{620} measured at fixed time points that occurred during the respective logarithmic phase and where a ''steady-state growth inhibition'' was reached. For yeast expressing the classical PKC isoforms (α or βI) estimation of drug effects was based on OD_{620} measurements at 65 h. For yeast expressing the novel PKCs (δ or η) estimation of drug effects was based on OD_{620} measurements at 45 h. For yeast expressing PKC-ζ estimation of drug effects was based on OD_{620} measurements at 45 h.

Growth inhibition values caused by 10^{-5} M PMA (arachidonic acid for PKC-ζ) on yeast expressing individual PKC isoforms are presented in Table 2 and were assumed to correspond to 100% growth inhibition reachable in the respective isoform. Effects of drugs were expressed as percentage of that growth inhibition value.

According to the maximal growth inhibition caused, phorbol esters tested were grouped into three groups. A first group, composed by PMA, PDD and PDB, caused the most marked growth inhibition in all PKC isoforms tested. In general, the maximal growth inhibition caused by PMA was higher on yeast expressing classical PKCs than on yeast expressing the novel PKCs. PDD and PDB caused maximal effects similar to those caused by PMA on all the responsive isoforms (Fig. 4). A second group, composed by PA, PDA, PMM and dPPA, showed to be inactive or much less active than PMA on PKC isoforms tested, causing a maximal growth inhibition smaller than that of PMA. However, in some isoforms, growth inhibition elicited by these compounds was higher than 50%, allowing estimation of EC_{50} values (Fig. 4). The third group, comprises MPMA and 4α-PMA, which even at the maximal concentration tested (10^{-5} M), were unable to reach 50% growth inhibition in any of the isoforms tested (Fig. 5). In yeast expressing the classical PKCs, these compounds even caused a marked growth stimulation (Fig. 5). Since stimulation of yeast growth was not observed in the absence of PKC expression, it was assumed to be an effect mediated by the PKC isoform expressed.

Table 3 shows EC_{50} values for phorbol esters in each of the PKC isoforms tested. According to the respective EC_{50} values, the rank order of potency obtained for the indicated phorbol ester was: for PMA, η>α>βI>δ; for PDD,

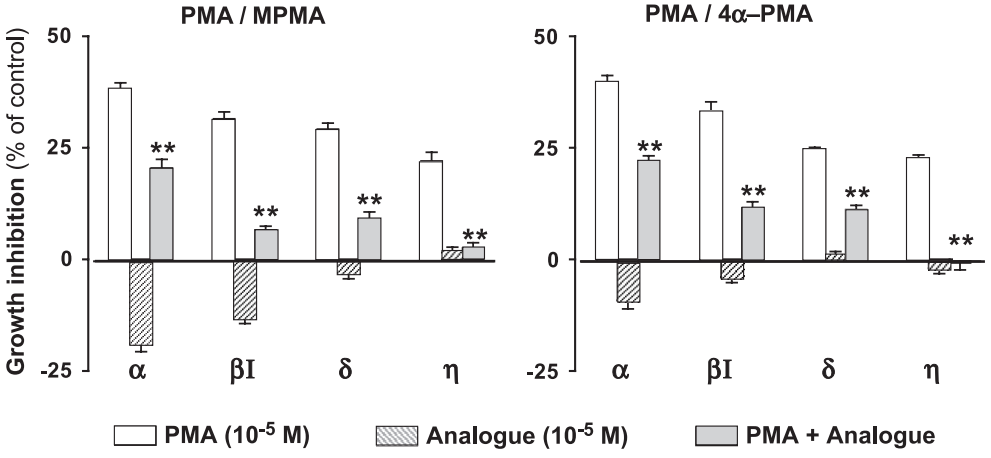


Fig. 5. Interaction experiments: effects of the PMA-analogues, MPMA and 4α-PMA, on PMA-induced growth inhibition of yeast expressing mammalian PKC-α, -βI, -δ or -η. Yeast cells expressing the indicated PKC isoform were incubated with phorbol esters or solvent (0.1% DMSO); growth was measured at 65 h (for classical PKCs; α and βI) or at 45 h (for novel PKCs; δ and η) incubation. PMA-analogues were tested alone and combined with PMA at the same concentration (10^{-5} M). Results are expressed as % of growth inhibition; 100% growth inhibition occurred when there was no increase on yeast growth during incubation; 0% growth inhibition was considered to occur when growth in the presence of drugs was identical to that in the presence of solvent; negative growth inhibition was considered to occur when growth in the presence of drugs was higher than that in the presence of solvent and was taken to indicate growth stimulation. Shown are means ± S.E.M. of 16–48 determinations. Significant differences from 10^{-5} M PMA alone: $^{**}P<0.01$ (unpaired Student's *t*-test).

Table 3
EC_{50} values of phorbol esters on individual PKC isoforms studied

Phorbol ester	EC_{50} (nM)				
	PKC-α	PKC-βI	PKC-δ	PKC-η	PKC-ζ[a]
PMA	112±18[#]	243±69[#]	574±37[#]	0.1±0.01	*ND*
PDD	42±4	67±16	51±10*	32±2*	*ND*
PDB	2740±372*,#	1040±107*,#	3530±178*,#	8±3	*ND*
PA	*ND*	*ND*	*ND*	6±1	*ND*
PDA	*ND*	*ND*	1190±97*,#	7400±768*	*ND*
PMM	*ND*	*ND*	1290±212*,#	5230±1101*	*ND*
dPPA	*ND*	3440±929*,#	*ND*	438±59*	*ND*

The EC_{50} values were considered the concentration of PKC activator that caused 50% of the growth inhibition caused by 10^{-5} M PMA (arachidonic acid for PKC-ζ). Phorbol esters were tested in a concentration range of 10^{-13}–10^{-5} M. Shown are means ± S.E.M. of 20 determinations. *ND*: nondeterminable (when the maximal response reached was lower than 50% growth inhibition). Significant differences: from PMA.

[a] On yeast expressing PKC-ζ, arachidonic acid caused a concentration-dependent growth inhibition with an EC_{50} of 205±33 nM (n=20).

* $P<0.05$; from PKC-η,

# $P<0.05$ (one way ANOVA, followed by Tukey's post hoc test).

η = α = δ = βI; for PDB, η>βI>α = δ; PDA and PMM showed to be active only in the novel PKCs (δ>η); dPPA activated βI and the η isoforms (η>βI), whereas PA was active only in the η isoform. For the indicated PKC isoform, phorbol esters can be ranked as following: for the α isoform, PDD>PMA>PDB; for the βI isoform, PDD>PMA>PDB>dPPA; for the δ isoform, PDD>PMA>PDA = PMM>PDB; for the η isoform, PMA>PA = PDB>PDD>dPPA>PMM = PDA (for definition of the rank order of potency, the sign > was applied only when the EC_{50} value of the compound placed at the left was significantly higher than the EC_{50} placed at the right of the sign; otherwise = was applied; $P<0.05$; unpaired Student's t-test). In PKC-ζ, all the phorbol esters were inactive (see Fig. 2).

3.3. Interaction experiments

The possibility that the ''inactive'' phorbol esters, MPMA and 4α-PMA, interfere with PKC activation caused by PMA was examined in this series of experiments. Results are expressed as percentage of growth inhibition (not standardized to the maximal effect caused by PMA). As before, 10^{-5} M PMA caused a more marked growth inhibition in yeast expressing classical PKCs than in yeast expressing novel PKCs (compare open columns of Fig. 5 with values in Table 2). MPMA and 4α-PMA were tested at the single concentration of 10^{-5} M, in the absence or in the presence of 10^{-5} M PMA. Results of such interactions on yeast expressing PKC-α, -βI, -δ or -η are shown in Fig. 5.

In the absence of PMA, MPMA and 4α-PMA stimulated growth of yeast expressing PKC-α or -βI (an effect more pronounced for MPMA). In the presence of 10^{-5} M PMA, these compounds reduced the PMA-induced growth inhibition in all the isoforms tested (Fig. 5). Since this effect also occurred on yeast expressing PKC-δ or -η, where MPMA and 4α-PMA when tested alone practically did not influence yeast growth, the reduction of the PMA effect cannot be explained simply by a balance between the PMA-induced growth inhibition and the growth stimulation induced by the analogue.

On the yeast phenotypic assay, PKC inhibitors reduce the PKC activator-induced growth inhibition and may cause

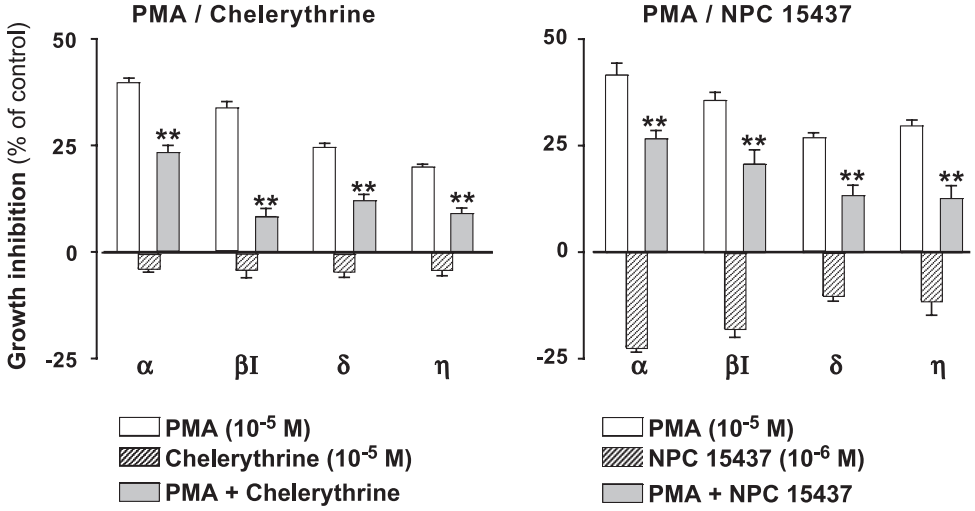


Fig. 6. Interaction experiments: effects of PKC inhibitors, chelerythrine and NPC 15437, on the PMA-induced growth inhibition of yeast expressing mammalian PKC-α, -βI, -δ or -η. Yeast cells expressing the indicated PKC isoform were incubated with compounds or solvent (0.1% DMSO) and growth was measured at 65 h (for classical PKCs; α and βI) or at 45 h (for novel PKCs; δ and η) incubation. Chelerythrine (10^{-5} M) or NPC 15437 (10^{-6} M) were tested alone and combined with PMA (10^{-5} M). Results are expressed as % of growth inhibition; 100% growth inhibition occurred when there was no increase on yeast growth during incubation; 0% growth inhibition was considered to occur when growth in the presence of drugs was identical to that in the presence of solvent; negative growth inhibition was considered to occur when growth in the presence of drugs was higher than that in the presence of solvent and was taken to indicate growth stimulation. Shown are means ± S.E.M. of 16–20 determinations. Significant differences from 10^{-5} M PMA alone: **$P<0.01$ (unpaired Student's t-test).

also, per se, a stimulation of yeast growth (Keenan et al., 1997; Saraiva et al., 2003). Therefore, effects of MPMA and 4α-PMA were compared to those exhibited by two known PKC inhibitors, chelerythrine (Herbert et al., 1990) and NPC 15437 (Petros et al., 1991; Sullivan et al., 1991, 1992). Effects of chelerythrine (10^{-5} M) and NPC 15437 (10^{-6} M; a higher concentration interfered with yeast growth in the absence of PKC expression) on the yeast growth, in the absence and in the presence of 10^{-5} M PMA, are presented in Fig. 6. Like MPMA and 4α-PMA, NPC 15437 also caused a marked stimulation of yeast growth and, like MPMA and 4α-PMA, both chelerythrine and NPC 15437 reduced the PMA-induced growth inhibition.

4. Discussion

In the present study, the mammalian PKC-α, -βI, -δ, -η, or -ζ were individually expressed in the same strain of *S. Cerevisiae*. Transformed yeasts were used to characterize, in detail and in vivo, the effects of a group of structurally close phorbol esters on each of these PKC isoforms. In *S. cerevisiae*, a PKC-related protein is also present but did not respond to phorbol esters, as predicted (Iwai et al., 1992; Ogita et al., 1990). Growth inhibition induced by phorbol esters occurred only when the mammalian PKC isoform was expressed and, therefore, was assumed to reflect activation of the PKC isoform expressed as previously described (Riedel et al., 1993a,b; Shieh et al., 1995, 1996). Under these conditions, the main methodological limitations of the in vivo assays (co-existence of several PKC isoforms and the presence of phorbol ester receptors other than PKC) were circumvented and the study of phorbol esters activities on each PKC isoform expressed was possible.

PMA was used as a standard PKC activator. In the present study, PMA activated classical and novel PKCs but not atypical PKCs, as observed previously in yeast expressing mammalian PKC isoforms (α: Riedel et al., 1993b; Shieh et al., 1995, 1996; βI: Riedel et al., 1993a; Shieh et al., 1996; γ, δ, η and ζ: Goode et al., 1994). According to the EC_{50} values, PMA seems to be much more potent on PKC-η than on PKC-α, -βI and -δ.

PDD and PDB are two other phorbol esters frequently used as PKC activators. Like PMA, they activated the classical and novel PKC isoforms tested, presenting an efficacy similar to that of PMA, i.e., their maximal response was similar to that caused by 10^{-5} M PMA. When compared to PMA, PDD showed a similar potency on all isoforms tested, suggesting that PDD may be a prototype of a potent and non-isoform-selective PKC activator. PDB was less potent than PMA on all the responsive isoforms and, like PMA, presented its highest potency on PKC-η. By similitude with the concepts of full and partial agonists, applicable to ligands for membrane-receptors (Kenakin, 1993), these PKC activators could be called "full activators".

The position of PMA and PDB in the rank order of potency, for PKC-α, -βI and -δ, differs from that presented in the rank order of affinity for these isoforms, reported in binding studies (Dimitrijevic et al., 1995). Although binding data indicated a lower affinity for PKC-βI than for PKC-δ (Dimitrijevic et al., 1995), we found that PMA was more potent on PKC-βI than on PKC-δ. Furthermore, PDB was more potent on PKC-βI than on PKC-α or -δ, whereas binding data showed that PDB has a lower affinity for PKC-βI than for PKC-α or -δ (Dimitrijevic et al., 1995). The observed differences on the rank order of potency and affinity may have a molecular basis. Studies, carried out in yeast expressing deletion mutants of mammalian PKC-α, showed that the lack of certain amino acids led to loss of activity without changing the affinity of ligands, leading to the conclusion that "...the binding of an activator to a specific PKC sequence may not necessarily reflect its role in the regulation of the PKC enzymatic activity" (Shieh et al., 1995). Therefore, a pharmacologic property like intrinsic activity (see Kenakin, 1993) may also be determinant for the activity of PKC ligands.

Affinity of PA, PDA, PMM and dPPA to PKC has been previously reported: [^{3}H]-PDB binding was decreased by PA (mouse skin: Delclos et al., 1980; mouse brain: Dunphy et al., 1980), by PDA (mouse skin: Delclos et al., 1980; mouse brain: Dunphy et al., 1980), by PMM (rat brain: Niedel et al., 1983) and by dPPA (Dimitrijevic et al., 1995). In the present study, PA, PDA, PMM and dPPA elicited, in the responsive isoforms, a maximal effect lower than that caused by the "full activators". Therefore, these compounds may be called "partial activators". Among them, PA presented selectivity for PKC-η, whereas PDA and PMM showed to be selective for novel PKCs, particularly for PKC-δ.

Previous in vitro studies suggested that dPPA is a selective PKC-βI activator (Dimitrijevic et al., 1995; Evans et al., 1991; Ryves et al., 1991). This putative selectivity has been questioned by an in vivo study, in which dPPA also activated PKC-α and -δ (Das et al., 1998). The present study also argues against the selectivity of dPPA for PKC-βI, because it activated PKC-η with a potency higher than that of PKC-βI, but no evidence was found that supports this claim (Das et al., 1998) that dPPA activates PKC-α and -δ. Furthermore, our observations are in agreement with the low affinity presented by dPPA on these isoforms (Dimitrijevic et al., 1995).

According to molecular modeling studies, the presence of a lipophilic domain at C-12 and/or at C-13 positions of the phorbol skeleton is considered a requirement for PKC activation (Jeffrey and Liskamp, 1986; Wender et al., 1988). This prediction was largely confirmed in binding studies (Delclos et al., 1980; Dimitrijevic et al., 1995; Dunphy et al., 1980) and the present study provides functional data supporting these predictions: the "full activators" have the largest substituents at C-12 and at C-13 of the phorbol esters tested, whereas the lower

efficacy of the ''partial activators'' may be related to their smaller substituents. Our results also suggest that reduction on the size of the lipophilic substituent decreases the potency of phorbol esters more markedly on classical than on novel PKC isoforms. The importance of an acetyl group at C-20 (like in dPPA) to obtain a selective activation of PKC-βI (Dimitrijevic et al., 1995) is not supported by the present results.

Molecular modeling studies also indicated that the presence, at C-4, of a methoxyl group (like in MPMA) or of an α-oriented hydroxyl group (like in 4α-PMA) causes loss of activity of PKC activators (Jeffrey and Liskamp, 1986; Wender et al., 1988). We further observed that MPMA and 4α-PMA failed to inhibit growth of yeast expressing individual classical and novel PKC isoforms and reduced the effect of the PKC activator, PMA. These effects of MPMA and 4α-PMA were similar to those caused by the two well known PKC inhibitors, chelerythrine (Herbert et al., 1990) and NPC 15437 (Petros et al., 1991; Sullivan et al., 1991, 1992), what suggest that MPMA and 4α-PMA may be acting like PKC inhibitors. A putative action of MPMA as PKC inhibitor may explain previous works that showed that, although MPMA was an inactive tumor promoter, it was able to reduce the PMA-induced tumor promotion (Slaga et al., 1980). Therefore, MPMA should not be considered an inactive phorbol ester, because by preventing effects of PKC activators it may present PKC mediated action and, therefore, the present study gives further support to the doubts concerning the use of MPMA as a negative control (Fürstenberger et al., 1982). The same may also hold true for 4α-PMA.

In conclusion, the present work shows in vivo, and in a system where only the response of a mammalian PKC isoform is revealed, that structurally close phorbol esters differ on their efficacy and potency to activate individual PKC isoforms. It is also presented functional data that can contribute for the definition of the structure–activity relationships of phorbol esters upon mammalian PKC. In addition, this study questions the use of some phorbol esters as negative controls, since the so-called ''inactive'' presented effects compatible to a PKC inhibition.

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