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Inhibition of Protein Kinase C by Synthetic Xanthone Derivatives

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Abstract—The modulatory activity of two xanthones (3,4-dihydroxyxanthone and 1-formyl-4-hydroxy-3-methoxyxanthone) on isoforms α, βI, δ, η and ζ of protein kinase C (PKC) was evaluated using an in vivo yeast phenotypic assay. Both xanthones caused an effect compatible with PKC inhibition, similar to that elicited by known PKC inhibitors (chelerythrine and NPC 15437). PKC inhibition caused by xanthones was confirmed using an in vitro kinase assay. The yeast phenotypic assay revealed that xanthones present differences on their potency towards the distinct PKC isoforms tested. It is concluded that 3,4-dihydroxyxanthone and 1-formyl-4-hydroxy-3-methoxyxanthone may become useful PKC inhibitors and xanthone derivatives can be explored to develop new isoform-selective PKC inhibitors.

Introduction

Protein kinase C (PKC) is a family of serine-threonine kinases with important roles in cellular functions such as growth, differentiation, tumor promotion and apoptosis.[1] PKC isoforms are grouped into at least three groups: the classical PKCs (cPKCs), which include the isoforms α, βI, βII and γ; the novel PKCs (nPKCs), which include the isoforms δ, ε, θ, and η; and the atypical PKCs (aPKCs), which include the isoforms ζ and λ/ι.[2,3] More recently, a fourth group of structurally distinct PKCs has been identified, the so-called PKC-related kinases (PRKs).[2,3] A new member of the PKC family, PKCμ/PKD has also been reported, but its inclusion remains controversial.[2]

Knowledge of specific roles attributable to individual PKC isoforms has been hampered by the lack of isoform-selective drugs and the search for selective drugs has been difficult because of several methodological limitations. Examples of these limitations are the coexistence of several isoforms in the cells used for in vivo assays, and the difficulties to reproduce in vitro the interactions occurring in vivo between PKC and other cellular constituents. These limitations can be circumvented using the yeast phenotypic assay. This is an in vivo assay, using yeast expressing an individual mammalian PKC isoform, based on the growth inhibition (reflecting an increase in the cell doubling time) which is proportional to the degree of PKC activation.[4,5] In the yeast phenotypic assay, PKC activators cause growth inhibition of transformed yeast[4,5] while PKC inhibitors block the growth inhibition caused by a PKC activator.[6,7]

Natural and synthetic xanthones have been reported to mediate several important biological activities namely anti-tumor,[8–12] anti-inflammatory,[13] anti-thrombotic[14] and neuropharmacological effects.[15–17] Recently, it has been suggested that these compounds may act, at least in part, by interacting with PKC, and cause effects compatible not only with PKC activation[18] but also with PKC inhibition. Prenylated xanthones were shown to be potent inhibitors of eukaryote kinases in vitro, including PKC[19] and norathyriol (1,3,6,7-tetrahydroxyxanthone) reduced the PMA-induced respiratory burst and aggregation, an effect ascribed to inhibition of PKC.[20]

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The modulatory activity of a series of twenty-two simple xanthones (9*H*-xanthen-9-ones) on PKC isoforms α and βI (cPKCs), δ and η (nPKCs) and ζ (aPKCs) were characterized in our laboratory using the yeast phenotypic assay. The majority of these compounds caused an effect compatible with PKC activation.[21] In the present study, we report that two of the xanthones tested (3,4-dihydroxyxanthone and 1-formyl-4-hydroxy-3-methoxyxanthone) caused effects compatible with PKC inhibition. The effects of these xanthones were compared with two established potent and selective PKC inhibitors (chelerythrine[22] and NPC 15437[23,24]). The two compounds presented differences on their potency towards the individual PKC isoforms tested. Furthermore, for some isoforms, they showed potencies even higher than those of the PKC inhibitors used. Therefore, 3,4-dihydroxyxanthone and 1-formyl-4-hydroxy-3-methoxyxanthone can be used as PKC inhibitors for characterization of PKC-mediated effects and for the development of new PKC isoform-selective inhibitors.

Results

Expression of mammalian PKC-α, -βI, -δ, -η and -ζ was confirmed by immunoblotting, of protein extracts of yeast cells, transformed with the gene of a single mammalian PKC isoform, grown in the presence of the transcription inducer (2% galactose). Expression of each of these PKC isoforms resulted in a single antigenic band, which co-migrated with the respective recombinant protein. Protein extracts of transformed yeast cells grown in the absence of galactose did not present antigenic bands (Fig. 1).

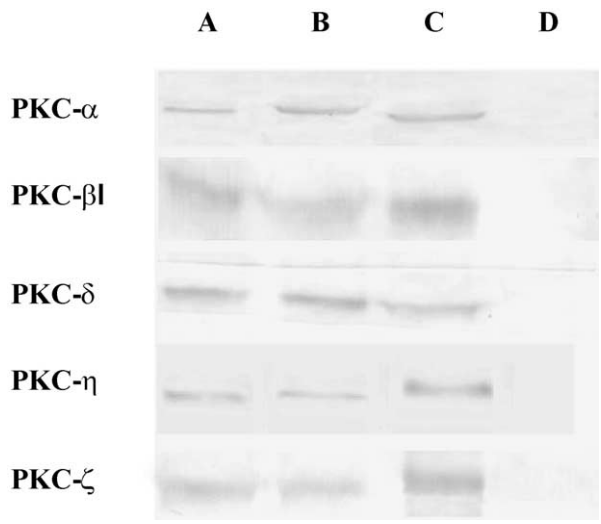

Figure 1. Immunodetection of PKC-α, -βI, -δ, -η and -ζ isoforms expressed in transformed *Saccharomyces cerevisiae* (CG379). Individual immunoblots are presented in a horizontal arrangement and were obtained from proteins extracts (~40 μg protein/lane) from cultures grown in selective medium in the presence of 2% galactose (lanes A and B; duplicate samples) or in the absence of 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).

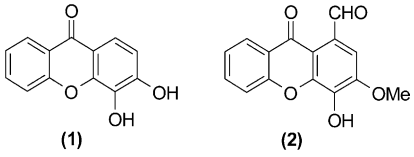

Figure 2. Chemical structure of 3,4-dihydroxyxanthone (**1**) and 1-formyl-4-hydroxy-3-methoxyxanthone (**2**).

Yeast phenotypic assay

PMA (considered a standard activator for the classical and novel PKC isoforms) was tested in yeast expressing PKC-α, -βI, -δ or -η, in concentrations up to 10^{-5} M (higher concentrations were not possible to test due to its low solubility in the culture medium). Since atypical PKC isoforms are not activated by phorbol esters,[25] arachidonic acid (up to 10^{-5} M) was used as the standard activator for PKC-ζ.[26] Effects of drugs were expressed as percentage of the maximal growth inhibition caused by the standard PKC activator (PMA for PKC-α, -βI, -δ, and -η or arachidonic acid for PKC-ζ; 100% growth inhibition was assumed to be that caused by 10^{-5} M of the respective PKC activator).

In the absence of galactose, neither PMA nor arachidonic acid altered yeast growth. The solvent used (DMSO; final concentration 0.1%) did not change yeast growth either in the presence or in the absence of galactose (not shown).

The influence of established PKC inhibitors (chelerythrine and NPC 15437) and of 3,4-dihydroxyxanthone (**1**) and 1-formyl-4-hydroxy-3-methoxyxanthone (**2**) (Fig. 2) on the growth of yeast expressing individual PKC isoforms was also studied. In the absence of galactose, chelerythrine, xanthones **1** and **2**, all tested in the concentration of 10^{-5} M, did not influence yeast growth. NPC 15437, in concentrations higher than 10^{-6} M, inhibited yeast growth. Therefore, to avoid these effects non-mediated by the expressed mammalian PKC isoform, 10^{-6} M was the maximal concentration of NPC 15437 tested when expression of mammalian PKC isoforms were induced (galactose present in the medium).

In the presence of galactose, PMA caused a concentration-dependent inhibition on growth of yeast expres-

Table 1. Yeast growth inhibition caused by 10^{-5} M PMA on the PKC isoforms studied

PKC isoforms	Growth inhibition caused by 10^{-5} M PMA
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; see Experimental for details). Each value represents the mean ± SEM of the indicated n determinations.
[a]Growth inhibition caused by 10^{-5} M arachidonic acid was 23.9 ± 0.7 (n = 56).

sing cPKCs or nPKCs isoforms. EC_{50} values were (nM) 99.4±10.1 (PKC-α), 214.7±48.0 (PKC-βI), 453.1±32.8 (PKC-δ) and 7.6±0.6 (PKC-η) ($n=64$), for the isoform indicated. In yeast expressing PKC-ζ, arachidonic acid, but not PMA, caused a concentration-dependent growth inhibition of yeast expressing this isoform, with an EC_{50} of 208.2±30.3 nM ($n=64$). Maximal values of growth inhibition caused by 10^{-5} M PMA (or arachidonic acid for PKC-ζ), on the PKC isoforms tested are presented on Table 1.

In the yeast phenotypic assay, a PKC inhibitor may be characterized by its capacity to block the growth inhibition caused by a PKC activator[6,7] and may cause, per se, a stimulation of yeast growth.[6] Therefore, chelerythrine, NPC 15437 and xanthones **1** and **2** were tested alone and combined with the appropriate PKC activator (PMA for PKC-α, -βI, -δ and -η; arachidonic acid for PKC-ζ).

On a first approach, chelerythrine, NPC 15437 and xanthones were tested alone and only on the maximal

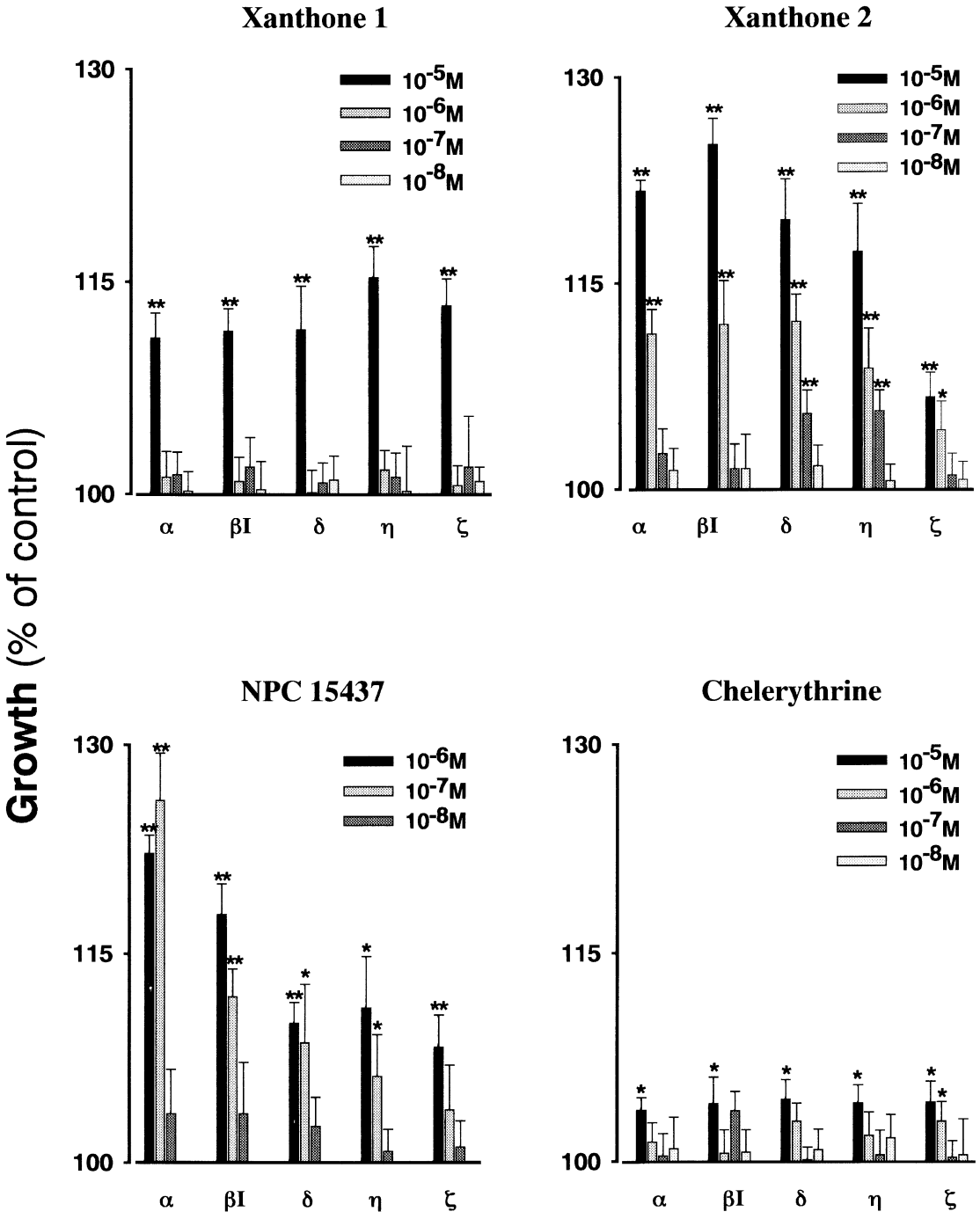

Figure 3. Effects of xanthones **1** and **2** and of the PKC inhibitors (chelerythrine and NPC 15437) on the growth of yeast expressing individual PKC isoforms (α, βI, δ, η or ζ). Yeast cells expressing the indicated mammalian PKC isoform were incubated with each compound at the concentration indicated or solvent (DMSO, 0.1% final concentration). Results are expressed as % of growth. Shown are means ± SEM of 16–24 determinations. Significantly different from growth in the presence of solvent: $^{*}P<0.05$, $^{**}P<0.001$ (paired Student's t test).

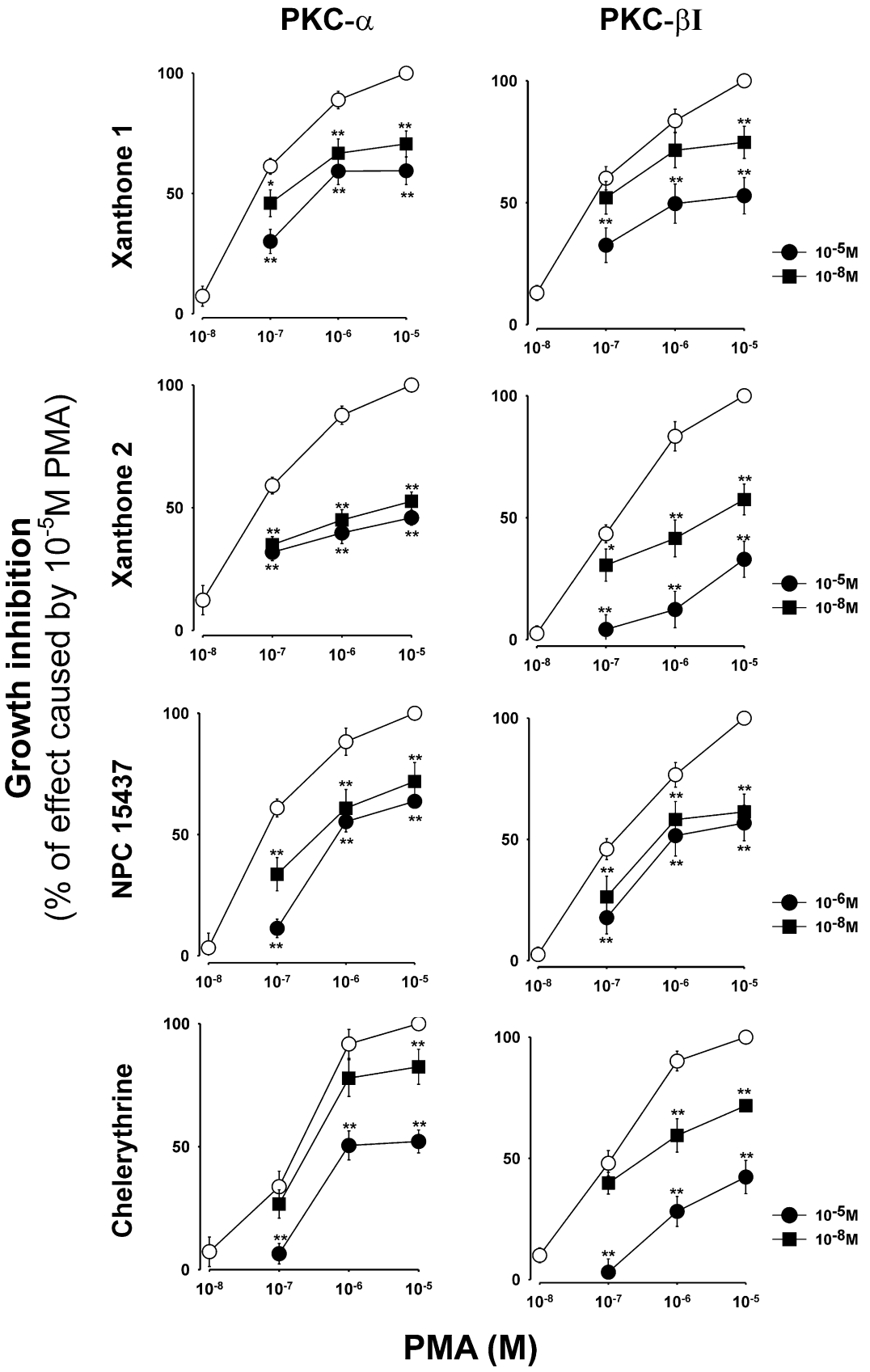

Figure 4. Interaction experiments on cPKCs (α and βI): concentration-response curves for PMA alone (open circles) and in the presence of 10^{-5} M xanthone **1**, xanthone **2**, chelerythrine and 10^{-6} M NPC 15437 (filled circles) or 10^{-8} M (filled squares). Results are expressed as % of the maximal effect caused by 10^{-5} M PMA. Shown are means ± SEM of 16–20 determinations. Significantly different from growth inhibition caused by PMA alone: $^{*}P < 0.05$, $^{**}P < 0.001$ (unpaired Student's *t* test).

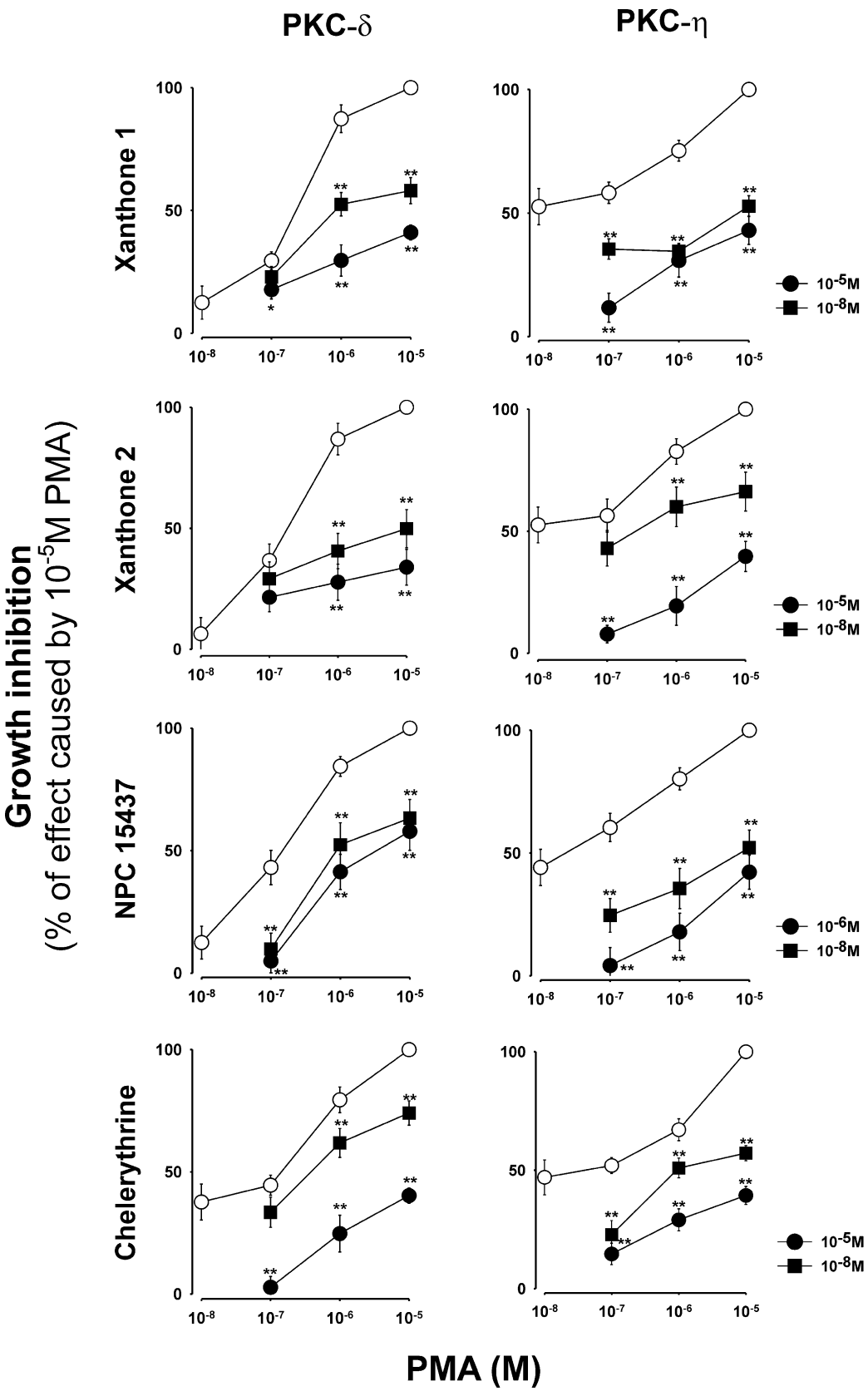

Figure 5. Interaction experiments on nPKCs (δ and η): concentration-response curves for PMA alone (open circles) and in the presence of 10^{-5} M xanthone **1**, xanthone **2**, chelerythrine and 10^{-6} M NPC 15437 (filled circles) or 10^{-8} M (filled squares). Results are expressed as % of the maximal effect caused by 10^{-5} M PMA. Shown are means ± SEM of 16–20 determinations. Significantly different from growth inhibition caused by PMA alone: $^{*}P < 0.05$, $^{**}P < 0.001$ (unpaired Student's *t* test).

concentration feasible under these experimental conditions: 10^{-5} M for chelerythrine and xanthones **1** and **2**; 10^{-6} M for NPC 15437. In general, both PKC inhibitors chelerythrine and NPC 15437, and xanthones **1** and **2** stimulated growth of yeast expressing the individual mammalian PKC isoforms (Fig. 3), although this growth stimulation was less marked with chelerythrine than with NPC 15437 or xanthones **1** or **2**. A decrease on concentration reduced the growth stimulation induced by these drugs. At 10^{-8} M, chelerythrine, NPC 15437, xanthones **1** and **2** failed to cause a significant stimulation of yeast growth (Fig. 3).

On a subsequent series of experiments, the ability of chelerythrine, NPC 15437, xanthones **1** and **2** to block the PKC activator-induced growth inhibition was investigated. These compounds were tested both at the maximal concentration they showed to be selective for PKC (10^{-5} M for chelerythrine and xanthones **1** and **2**; 10^{-6} M for NPC 15437) and at 10^{-8} M (concentration at which all failed to significantly change growth of transformed yeast). Effects of chelerythrine, NPC 15437 and xanthones **1** and **2** on the growth inhibition caused by the PKC activator PMA on yeast expressing PKC-α or -βI and on yeast expressing PKC-δ or -η are shown in Figs. 4 and 5, respectively. Figure 6 shows the effects of chelerythrine, NPC 15437 and xanthones **1** and **2** on the growth inhibition caused by arachidonic acid on yeast expressing PKC-ζ.

In general, both chelerythrine and NPC 15437 reduced the growth inhibition caused by the PKC activator, causing a rightward shift on the concentration-response curve of the PKC activator (compare open and filled symbols in lower panels of Figs. 4–6). The rightward shift was, generally, concentration-dependent being the displacement caused by 10^{-8} M less pronounced than that caused by 10^{-5} M chelerythrine or by 10^{-6} M NPC 15437 (compare filled squares and circles in lower panels of Figs 4–6). Xanthones **1** and **2** also caused, in general, a rightward shift of the concentration-response curve of the PKC activator (see upper panels of Figs 4–6). As observed with chelerythrine and NPC 15437, the rightward shift was, in general, concentration-dependent being the displacement caused by 10^{-8} M less pronounced than that caused by 10^{-5} M xanthones **1** or **2** (compare filled squares and circles in upper panels of Figs 4–6). However, in some cases (xanthone **1** on PKC-ζ, xanthone **2** on PKC-α and-δ, NPC 15437 on PKC-βI and-δ), no concentration-dependence was found, being the rightward shift identical for the highest and the lowest concentration of PKC inhibitor tested ($P > 0.05$; unpaired Student's t test).

In order to compare the potency of the PKC inhibitors to that of xanthones **1** and **2** in blocking the effect of PKC activators, EC_{50} ratios of the PKC activator, obtained in the presence and in the absence of PKC inhibitors or xanthones **1** or **2**, were calculated (EC_{50} is the concentration of the PKC activator that caused half of the growth inhibition caused by 10^{-5} M PMA; arachidonic acid for PKC-ζ). Because the highest concentration of NPC 15437 and xanthones **1** and **2** caused

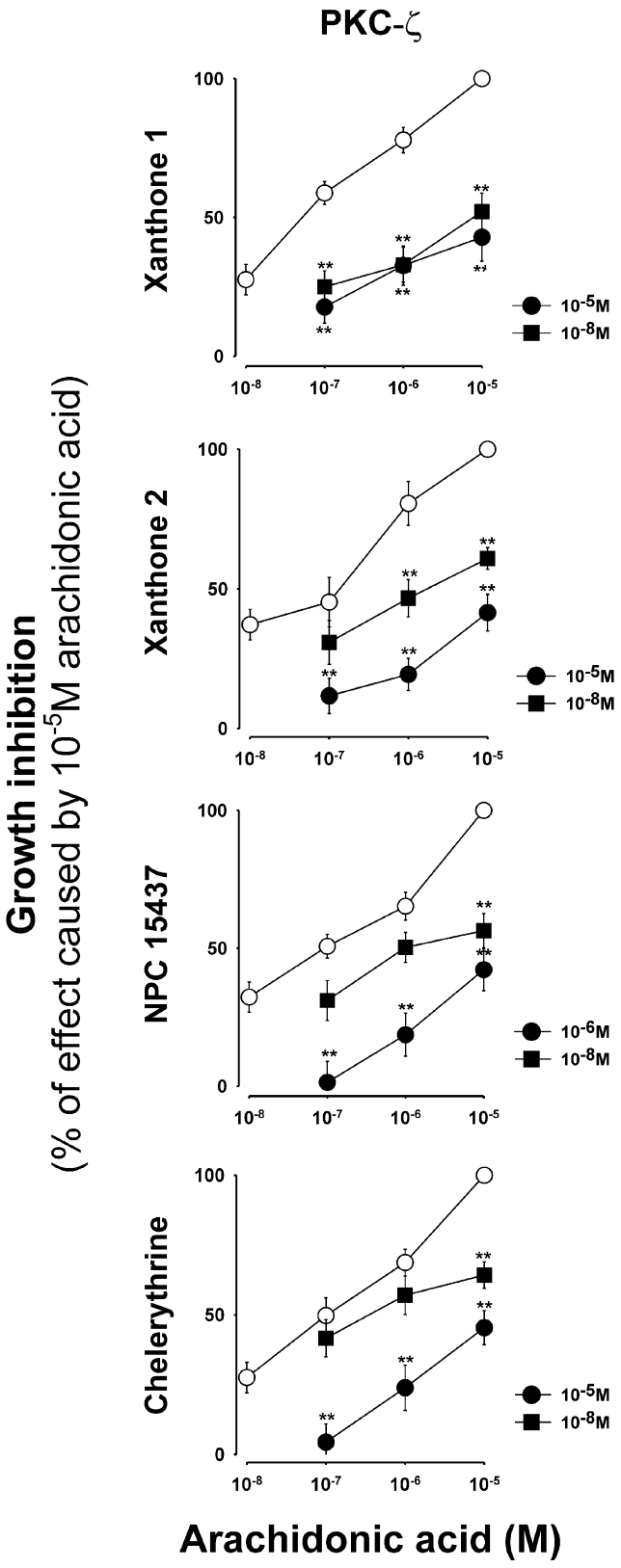

Figure 6. Interaction experiments on aPKC (ζ): concentration–response curves obtained for arachidonic acid alone (open circles) and in the presence of 10^{-5} M xanthone **1**, xanthone **2**, chelerythrine and 10^{-6} M NPC 15437 (filled circles), or 10^{-8} M (filled squares). Results are expressed as % of the maximal effect caused by 10^{-5} M of arachidonic acid. Shown are means ± SEM of 16–20 determinations. Significantly different from growth inhibition caused by arachidonic acid alone: $^{*}P < 0.05$, $^{**}P < 0.001$ (unpaired Student's t test).

Table 2. EC_{50} ratios for xanthones **1** and **2**, NPC 15437 and chelerythrine on the individual PKC isoforms tested

Compound	EC_{50} ratio[a]				
	PKC-α	PKC-βI	PKC-δ	PKC-η	PKC-ζ
Xanthone **1**	$1.8\pm0.2^{*\S}$	$0.8\pm0.1^{*\S}$	$4.5\pm0.8^{*\S}$	600.0 ± 25.4	$40.1\pm1.8^{\S}$
Xanthone **2**	79.2 ± 6.9	$28.7\pm2.4^{\dagger}$	$24.0\pm1.6^{\dagger}$	$20.0\pm2.8^{\ddagger\dagger}$	$10.4\pm1.1^{\ddagger\dagger}$
NPC 15437	$5.1\pm0.5^{*\S}$	$3.7\pm0.3^{*\S}$	$2.4\pm0.1^{*\S}$	556.0 ± 44.7	$4.5\pm0.1^{\ddagger\S}$
Chelerythrine	$1.9\pm0.3^{*\S}$	$2.9\pm0.3^{*\S}$	$1.3\pm0.1^{*\S}$	$63.2\pm1.7^{\ddagger}$	$1.4\pm0.2^{\ddagger\S}$

The EC_{50} values were considered the concentration of PKC activator that caused half of the growth inhibition caused by 10^{-5} M of PMA (arachidonic acid for PKC-ζ). Shown are means ± SEM of 16–20 determinations. Significant differences: from xanthone **2**, $^{*}P<0.05$; from xanthone **1**, $^{\ddagger}P<0.05$; from PKC-α, $^{\dagger}P<0.05$; from PKC-η, $^{\S}P<0.05$ (one way ANOVA, followed by Tukey's post-hoc test).
[a]EC_{50} ratio = EC_{50} (PKC activator + 10^{-8} M compound)/EC_{50} (PKC activator).

growth stimulation, only ratios obtained in the presence of 10^{-8} M (which did not cause a significant stimulation of growth) were used for comparisons (Table 2). According to the EC_{50} ratios obtained, chelerythrine, NPC 15437 and xanthones **1** and **2** presented differences on their potencies towards the PKC isoforms tested and the following order of potencies for the compounds studied was proposed: for chelerythrine, PKC-η > -βI > -α > -ζ = -δ; for NPC 15437, PKC-η > -α = -βI = -ζ > -δ; for xanthone **1**, PKC-η > -ζ > -δ > -α = -βI and for xanthone **2**, PKC-α > -βI = -δ = -η > -ζ (for definition of the rank order of potency, the signal > was applied only when the EC_{50} ratio for the isoform placed at the left was significantly higher than that of the isoform placed at the right of the signal; otherwise = was applied; $P<0.05$; unpaired Student's t test). Like NPC 15437 and chelerythrine, xanthone **1** presented an higher EC_{50} ratio on PKC-η. On the other hand, the EC_{50} ratios obtained with xanthone **2** on the different isoforms tested did not differ so markedly, although it was shown to be higher on PKC-α than on other isoforms. Xanthone **1** was the compound which presented the highest EC_{50} ratio on PKC-η and -ζ, whereas xanthone **2** showed the highest EC_{50} ratio on PKC-α, -βI and -δ.

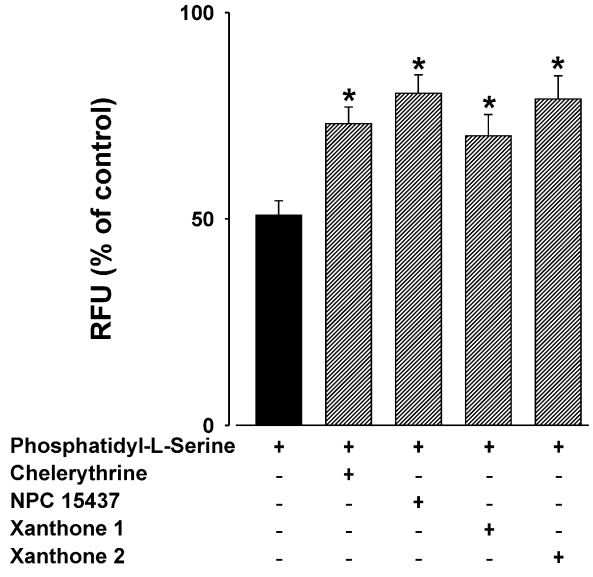

Figure 7. In vitro kinase assay using a purified rat brain PKC enzyme: effects of chelerythrine, NPC 15437, xanthone **1** and xanthone **2** (all tested at the concentration of 10^{-8} M). Relative fluorescence units (RFU) were measured in samples without enzyme (100%; control) or with PKC, which caused a decrease on RFU (see Experimental for details). Phosphatidyl-L-serine, the recommended PKC activator, was present in all samples; drugs or solvent, were tested in the absence or in the presence of PKC. Effects of drugs was expressed as % of RFU obtained in the absence of PKC. Shown are means ± SEM of four assays (8 assays for phosphatidyl-L-serine alone). Significantly different from solvent (phosphatidyl-L-serine alone): $^{*}P<0.05$ (one way ANOVA, followed by Tukey's post-hoc test).

In vitro kinase assay

To directly test if xanthones **1** and **2** were interacting with PKC, their effects were studied in vitro, on purified rat brain PKC. The test used is based on the ability of PKC inhibitors to revert the decrease on the relative fluorescence units (RFU) caused by the recommended endogenous PKC activator phosphatidyl-L-serine (which is assumed to reflect inhibition of the PKC catalytic activity). Xanthones **1** and **2**, chelerythrine and NPC 15437 were used on the lowest concentration tested (10^{-8} M), the same used to obtain the EC_{50} ratios presented on Table 2. Results are shown in Figure 7. Xanthones **1** and **2** were able to revert the effect of the endogenous PKC activator being the effects similar to those caused by chelerythrine and NPC 15437.

Discussion

The yeast phenotypic assay, which uses transformed yeast expressing individual mammalian PKC isoforms, has been proposed as an alternative rapid and simple in vivo assay for the screening of potential PKC modulators. In the present study, the mammalian PKC-α, -βI, -δ, -η or -ζ isoforms were expressed in the same yeast strain. According to previous studies,[21] exposure of transformed yeast expressing a mammalian PKC isoform to a PKC activator, caused a concentration-dependent inhibition of yeast growth, reflecting an increase in the cell doubling time. The inhibition of yeast growth caused by the PKC activator was assumed to be due to its interaction with the PKC isoform expressed, because it did not occur when transformed yeast cells were incubated in medium lacking the expression inducer, galactose. Confirmation that mammalian PKC isoforms were not expressed when galactose was not added to the medium was obtained by immunobloting. PMA failed to influence growth of yeast expressing PKC-ζ, confirming the well known inactivity of phorbol esters on isoforms of the atypical PKC family[25] and provided additional evidence that the growth inhibition caused by

PMA, in this assay, is due to PKC activation. Therefore, in the present experimental conditions, growth inhibition of yeast expressing an individual mammalian PKC isoform caused by the known PKC activators was assumed to reflect PKC activation.

Absolute values of growth inhibition caused by the PKC activators used (PMA or arachidonic acid) differ among the expressed PKC isoforms. These differences may be ascribed to variations on the degree of expression of the mammalian PKC isoform or to differences on the catalytic potential of the expressed isoform. The later possibility is supported by results obtained in vitro that showed that identical amounts of PKC isoforms, in the presence of the same concentration of a PKC activator, led to different degrees of phosphorylation of a given substract.[27] In order to avoid the influence of different absolute values on data interpretation, growth inhibition caused by PKC activators was standardized and, for each isoform, growth inhibition caused by a maximal concentration of PKC activator assumed to be the 100% of growth inhibition reachable under the present experimental conditions and EC_{50} values estimated afterwards.

PKC inhibitors, like chelerythrine and NPC 15437, caused, per se, a stimulation of yeast growth. A similar effect was reported to occur with bryostatin 1 on yeast expressing PKC-γ,[6] an effect probably due to blockade of the PKC activation caused by endogenous activators. Xanthones **1** and **2**, like NPC 15437, also stimulated growth of yeast expressing mammalian PKC isoforms. Nevertheless, chelerythrine, described as a potent PKC inhibitor, only marginally changed growth, what questions the use of growth stimulation as an index of potency for PKC inhibitors. Reasons for the lack of correlation between potency of PKC inhibitors and their ability to increase yeast growth are, at present, not fully understood, but may rely on the different site of interaction between these inhibitors and PKC: NPC 15437 interacts with the C1 region of the regulatory domain[23,24] whereas chelerythrine interacts with the ATP region of the catalytic domain.[22] Xanthones **1** and **2**, when tested alone, also stimulated yeast growth, with a pattern of effects similar to those of NPC 15437. This resemblance may indicate that these xanthones interact with PKC at the same domain of NPC 15437.

Blockade of the PKC activators-induced growth inhibition by established PKC inhibitors has been previously reported to reflect inhibition of the mammalian PKC isoform expressed.[6,7] Under these conditions, PKC inhibitors caused a rightward shift of the concentration-response curve for the PKC activator. The rightward shift was estimated by the ratio between the EC_{50} of the PKC activator obtained in the presence and in the absence of the PKC inhibitor. The obtained EC_{50} ratios were used as an index of the potency of the PKC inhibitor on the mammalian PKC isoform expressed.

Chelerythrine and NPC 15437 blocked the growth inhibition caused by PMA (PKC-α, -βI, -δ and -η) or by arachidonic acid (PKC-ζ). Xanthones **1** and **2** also blocked PKC activation caused by PMA or by arachidonic acid. Comparison of the potency was based on the comparison of EC_{50} ratios caused by concentrations of chelerythrine, NPC 15437, xanthone **1** and xanthone **2** that, per se, did not stimulate yeast growth. Under these conditions xanthone **1** caused a pattern of effects similar to that of NPC 15437: both xanthone **1** and NPC 15437 were very potent upon PKC-η and presented a low potency upon PKC-α, -βI and -δ. Chelerythrine presented also a marked potency on PKC-η, although it was less potent than xanthone **1** and NPC 15437. The high potency of xanthone **1** on PKC-ζ must also be emphasised. On PKC-ζ, xanthone **1** seems to be much more potent than NPC 15437 and chelerythrine. Xanthone **2** did not share the pattern of effects presented by NPC 15437 and chelerythrine, but presented the highest potency on PKC-α, -βI and -δ.

Like other PKC inhibitors, differences on the potency of xanthones **1** and **2** to block the effect of PKC activators may be influenced by their ability to reach their intracellular receptor (PKC) in mammalian cells. The yeast phenotypic assay is an in vivo assay, and factors that influence the access of compounds to intracellular receptors in yeast cells are similar to those present in more complex eukariotic cells. Therefore, the activity showed by xanthones **1** and **2** should reflect their potency in vivo.

Confirmation that, xanthones **1** and **2** blocked the effect of PKC activators on yeast growth by interacting with PKC was obtained using an in vitro kinase assay. At the lowest concentration used on the yeast phenotypic assay, xanthones **1** and **2**, like NPC 15437 and chelerythrine, inhibited the PKC catalytic activity, an effect similar to that caused by the established PKC inhibitors.

Previous studies showed that xanthones may act as PKC activators.[21] The present study extends this observation and shows that xanthone derivatives may also act as potent PKC inhibitors. Given the implication of PKC activation in carcinogenesis and other human diseases, PKC inhibition have become a major research target[28] and xanthone derivatives may become an important family to look for potent and selective PKC inhibitors.

Conclusions

This study shows, by using in vitro and in vivo assays, that simple xanthones can act as inhibitors of mammalian α, βI, δ, η and ζ PKC isoforms. The potencies presented by 3,4-dihydroxyxanthone and 1-formyl-4-hydroxy-3-methoxyxanthone on different PKC isoforms suggest that xanthones may be an important group to look for potent and isoform-selective PKC inhibitors.

Experimental

Chemistry

Purifications of compounds were performed by column chromatography; using Merck silica gel 60 (0.50–0.20 mm)

and preparative thin layer chromatography (tlc), using Merck silica gel 60 (GF_{254}). Melting points were obtained in a Köfler microscope and are uncorrected. IR spectra were recorded on a Perkin Elmer 257 in KBr. ^1H and ^{13}C NMR spectra were taken in $CDCl_3$ or DMSO-d_6 at room temperature, on Bruker AC 200 instrument. Chemical shifts are expressed in δ (ppm) values relative to tetramethylsilane (TMS) as an internal reference. MS spectra were recorded as EI (Electronic Impact) mode on a Hitachi Perkin–Elmer.

The synthesis of the following compounds have been carried out as follow.

3,4-Dihydroxyxanthone (1). This compound was obtained by demethylation of 3,4-dimethoxyxanthone according to the procedure described.[29] Mp >330 °C, chloroform (240–241 °C, ethanol/aqueous;[30] 238–240 °C, methanol[31]); υ_{max} (cm^{-1}) KBr: 3527, 3391, 3088, 1593, 1459, 1340, 1229, 1056, 756; ^1H NMR (DMSO-d_6, 200.13 MHz) δ: 8.14 (1H, dd, J = 8.1 and 1.6 Hz, H-8), 7.81 (1H, ddd, J = 8.6, 6.9 and 1.7 Hz, H-6), 7.62 (1H, dd, J = 8.6 and 0.9 Hz, H-5), 7.56 (1H, d, J = 8.6 Hz, H-1), 7.42 (1H, ddd, J = 8.1, 6.9 and 0.9 Hz, H-7), 6.93 (1H, d, J = 8.6 Hz, H-2); ^{13}C NMR (DMSO-d_6, 50.03 MHz) δ: 175.3 (C-9), 155.5 (C-4b), 151.6 (C-3), 146.4 (C-4a), 134.8 (C-6), 132.7 (C-4), 125.9 (C-8), 124.0 (C-7), 120.8 (C-8a), 118.0 (C-5), 116.6 (C-1), 114.7 (C-9a), 113.2 (C-2); MS m/z (rel int): 230 (3, $[M+2]^{+\cdot}$), 229 (22, $[M+1]^{+\cdot}$), 228 (100, $[M^{+\cdot}]$), 200 (14), 171 (9), 126 (10), 115 (12), 100 (8), 77 (5), 63 (7).

1-Formyl-4-hydroxy-3-methoxyxanthone (2). This compound was obtained according to the procedure described.[32]

General methods for the in vivo yeast phenotypic assay

Yeast nitrogen base was from DIFCO (Merck Portugal, Lisboa, Portugal). 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). Acid-washed glass beads, antibodies to PKC-α, PKC-βI, PKC-δ, PKC-η and PKC-ζ, aprotinin, arachidonic acid sodium salt, chelerythrine cloride, *R*-2,6-diamino-*N*-[[1-(1-oxotridecyl)-2-piperidinyl]methyl]-hexanamide dihydrochloride (NPC 15437 dihydrochloride), leupeptin, pepstatin A, phenylmethylsulfonyl fluoride, phorbol 12-myristate 13-acetate (PMA) were from Sigma Aldrich (Sintra, Portugal). All other chemicals used were of analytical grade.

Yeast transformation and cell cultures. Constructed yeast expression plasmids YEp52 and YEp51, encoding the 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* 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.[33] To ensure the 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 in leucine free-medium, with slow shaking, at 30 °C. 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.

Cell lysis and immunoblotting. Cell lysis was performed basically as described.[21] The protein concentration was determined using a kit for protein quantification (*Coomassie® Protein Assay Reagent Kit*, Pierce, Biocontec, Lisbon, Portugal). 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, Barcelona, Spain).

Yeast phenotypic assay. Transformed yeast cultures were incubated in leucine-free 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, with slow shaking at 30 °C, either in the presence of drugs or solvent (DMSO 0.1%; final concentration). Growth was monitored by determining the OD_{620} using a plate reader (BioRad Benchmark Microplate Reader; Hercules, CA, USA). In preliminary experiments, growth curves for individual isoforms were determined and the duration of the logarithmic and stationary phases identified. Estimation of drug effects was based on OD_{620} measurements at fixed time points (at 65 h for

cPKC isoforms or at 48 h incubation for nPKC and aPKC), times occurring during the respective logarithmic phase and where a 'steady-state growth inhibition' (period of time during which maximal inhibition of growth was reached and remained constant or changed only slightly) was reached. In individual experiments, OD_{620} was routinely monitored for up to 100 h to confirm whether the 'fixed time points' chosen were appropriate for all the compounds studied (PMA or arachidonic acid). The difference between the 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 parallel experiments in the presence of solvent; it was further transformed into growth inhibition by subtracting that value from 100. Because growth inhibition caused by a maximal concentration of the standard PKC activator varied between isoforms, 100% growth inhibition was assumed to be that caused by 10^{-5} M PMA (or arachidonic acid for PKC-ζ), in order to standardize the maximal inhibition reachable; 0% growth inhibition would occur when growth in the presence of drugs was identical to that in the presence of solvent. Effects of PKC activators were expressed as percentage of that effect.

For interaction experiments, a single concentration of PKC inhibitors or xanthones **1** or **2** were added to PMA (10^{-8}–10^{-5} M; arachidonic acid for PKC-ζ). Concentration-response curves for the PKC activator alone in the absence or in the presence of PKC inhibitors or xanthones **1** or **2**, were obtained and the concentration of PKC activator that caused 50% growth inhibition (EC_{50}) determined. For each compound the EC_{50} ratios [EC_{50} (PKC activator + 10^{-8} M compound)/EC_{50} (PKC activator)] were calculated on each PKC isoform tested.

In vitro kinase assay

The direct interaction between the compounds tested (PKC inhibitors and xanthones **1** and **2**) with PKC was studied in vitro using the IQ™ PKC Assay Kit—Pseudosubstrate Peptide Substrate from Pierce Biotechnology (PACI, Lisboa, Portugal). The assay was performed in agreement with the kit procedure for 96-well plate format. Xanthones **1** and **2**, and the standard PKC inhibitors (chelerythrine and NPC 15437) were tested at the concentration of 10^{-8} M. Purified PKC enzyme standard from rat brain (containing a mixture of α, β and γ isoforms) was from Pierce Biotechnology (PACI, Lisboa, Portugal); it was used at the final concentration of 0.01 Units. Reaction mixtures containing the endogenous PKC activator phosphatidyl-L-serine, were incubated for 90 min and fluorescence intensity determined using a microplate spectrofluorometer SPECTRAmax®GEMINI XS (Molecular Devices Corporation, Sunnyvale, CA, USA). In the presence of PKC, there was a decrease on relative fluorescence units (RFU), reflecting a phosphorylation of the fluorescent substrate. In the presence of PKC inhibitors, substrate phosphorylation is reduced causing an increase on RFU. RFU in samples containing no PKC were considered as 100%. Effects of drugs were expressed as percentage of that value.

Statistical analysis. Results are given as arithmetic means ± SEM 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 ANOVA, followed by Tukey's post-hoc test. A P value less than 0.05 was taken to be statistically significant.

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