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Synthesis and In Vivo Modulatory Activity of Protein Kinase C of Xanthone Derivatives

Lucília Saraiva,[a,b] Paula Fresco,[a] Eugénia Pinto,[b] Emília Sousa,[c] Madalena Pinto[c] and Jorge Gonçalves[a,*]

[a]*Serviço de Farmacologia, Faculdade de Farmácia, Universidade do Porto, rua Aníbal Cunha, 164, 4050-047 Porto, Portugal*
[b]*Serviço de Microbiologia, Faculdade de Farmácia, Universidade do Porto, rua Aníbal Cunha, 164, 4050-047 Porto, Portugal*
[c]*Serviço de Química Orgânica, Faculdade de Farmácia, Universidade do Porto, rua Aníbal Cunha, 164, 4050-047 Porto, Portugal*

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Abstract—The modulatory activity of a series of 20 simple xanthones on isoforms α, βI, δ, η and ζ of protein kinase C (PKC) was evaluated using an in vivo yeast phenotypic assay. Hydroxy and/or methoxyxanthones were synthesised. The majority of these compounds caused an effect compatible with activation of PKC and some showed to be more effective than the standard PKC activator (PMA or arachidonic acid). The xanthones tested differ in their efficacy and potency towards individual PKC isoforms and some showed higher selectivities for PKC-δ, -η or -ζ, suggesting that xanthone derivatives can become valuable research tools to elucidate the physiological roles of these isoforms.

Introduction

Protein kinase C (PKC) is a multifamily of serine-threonine kinases. According to their primary structure and cofactor requirements, PKC isoforms are grouped into at least three families: the classical PKCs (cPKCs), which include the isoforms α, βI, βII and γ; the novel (nPKCs), which include the isoforms δ, ε, θ, and η; and the atypical PKCs (aPKCs), which include the isoforms λ and ζ.[1,2] Important roles in cellular functions such as growth, differentiation, tumor promotion and apoptosis[3,4] have been ascribed to PKC. The knowledge of specific roles attributable to individual PKC isoform has been hampered by the lack of isoform-selective drugs. Similarly, the search for selective drugs has also been prevented by the lack of an in vivo method in which effects of drugs on a single mammalian PKC isoform can be tested. The in vitro assays use single isoforms, which are, nevertheless, difficult to isolate and purify. Furthermore, in this type of assays it is not possible to fully reproduce the interactions occurring in vivo between PKC and cellular constituents. These methodological difficulties can be circumvented by using the yeast phenotypic assay. This is an in vivo assay, based on the growth inhibition (reflecting an increase in the cell doubling time) of yeast expressing an individual mammalian PKC isoform, which is proportional to the degree of PKC activation.[5,6]

Natural and synthetic xanthones have been reported to mediate several important biological activities namely anti-tumor,[7–11] anti-inflammatory,[12] anti-thrombotic[13] and neuropharmacological effects.[14–16] Recently, some studies have suggested that these compounds may act, at least in part, by interacting with PKC. Prenylated xanthones have been shown to interact with eukaryote kinases in vitro, namely PKC.[17,18] Norathyriol (1,3,6,7-tetrahydroxyxanthone) reduced the PMA-induced respiratory burst and aggregation, an effect ascribed to a PKC inhibition.[19] Furthermore, 1,7-dihydroxyxanthone can mimick the induction of neuroblastoma cell differentiation caused by PKC activators, such as PMA, an effect blocked by PKC inhibitors.[20]

In the present study, the effects of a series of twenty simple xanthones (9*H*-xanthen-9-ones) on PKC isoforms α and βI (cPKCs), δ and η (nPKCs) and ζ (aPKCs) were characterized using the yeast phenotypic assay. With the exception of the xanthone itself, all xanthones were obtained by synthesis by our group. The criteria for the design of the compounds was the molecular rigidity associated with the xanthone nucleus

*Corresponding author. Tel.: +351-22-2078-932; fax: +351-22-2078-969; e-mail: jorge.goncalves@ff.up.pt

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and the absence of groups with the possibility of conformational isomerism. All the compounds are mono or dioxygenated in one or in the two aromatic rings (Table 1).

Our results show that these simple xanthones interact with PKC causing an effect compatible with a PKC activation. This screening also revealed that these compounds differ on their efficacy and potency to activate individual PKC isoforms and some showed effects higher than the standard PKC activators (PMA for cPKCs and nPKCs and arachidonic acid for aPKCs), what can be explored to develop new selective PKC activators.

Results

Expression of mammalian PKC-α, PKC-βI, PKC-δ, PKC-η and PKC-ζ was confirmed by immunoblotting, using 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).

A wide range of concentrations of PMA (considered as a standard activator for the classical and novel PKC isoforms) was tested in yeast expressing PKC-α, PKC-βI, PKC-δ and PKC-η. Concentrations higher than 10^{-5} M were not possible to test due to its low solubility in the medium. Since atypical PKC isoforms are not activated by phorbol esters,[21] arachidonic acid was used as the standard activator for PKC-ζ.[22]

In the presence of galactose, PMA caused a concentration-dependent inhibition on the growth of yeast expressing individual cPKCs or nPKCs isoforms, with IC_{50} values (nM) of 112±18 (PKC-α), 243±69 (PKC-βI), 574±37 (PKC-δ) and 0.1±0.01 (PKC-η) ($n=20$). In yeast expressing PKC-ζ, arachidonic acid, but not PMA, caused a concentration-dependent inhibition on the growth of yeast expressing this isoform, with an IC_{50} of 205±33 nM ($n=20$).

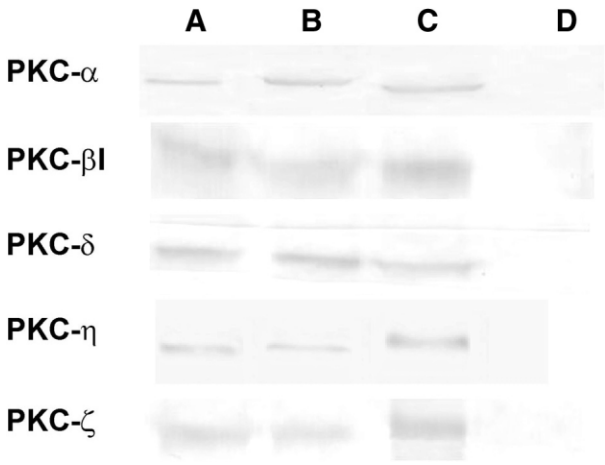
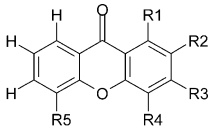


Figure 1. Immunodetection of PKC-α, -βI, -δ, -η and -ζ isoforms expressed in transformed *Saccharomyces cerevisiae* (CG379). Individual immunoblots are presented in a horizontal arrangement and were obtained from proteins extracted from cultures grown in selective medium in the presence of 2% galactose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

Table 1. Chemical structures of xanthone derivatives



Compound		R1	R2	R3	R4	R5
1	Xanthone	H	H	H	H	H
2	1-Hydroxyxanthone	OH	H	H	H	H
3	1-Methoxyxanthone	OCH_3	H	H	H	H
4	2-Hydroxyxanthone	H	OH	H	H	H
5	2-Methoxyxanthone	H	OCH_3	H	H	H
6	3-Hydroxyxanthone	H	H	OH	H	H
7	3-Methoxyxanthone	H	H	OCH_3	H	H
8	4-Hydroxyxanthone	H	H	H	OH	H
9	4-Methoxyxanthone	H	H	H	OCH_3	H
10	1,2-Dihydroxyxanthone	OH	OH	H	H	H
11	1,2-Dimethoxyxanthone	OCH_3	OCH_3	H	H	H
12	2,3-Dihydroxyxanthone	H	OH	OH	H	H
13	2,3-Dimethoxyxanthone	H	OCH_3	OCH_3	H	H
14	3,4-Dimethoxyxanthone	H	H	OCH_3	OCH_3	H
15	3,5-Dihydroxyxanthone	H	H	OH	H	OH
16	3,5-Dimethoxyxanthone	H	H	OCH_3	H	OCH_3
17	3-Hydroxy-4-Methoxyxanthone	H	H	OH	OCH_3	H
18	3-Hydroxy-5-Methoxyxanthone	H	H	OH	H	OCH_3
19	4-Hydroxy-3-Methoxyxanthone	H	H	OCH_3	OH	H
20	1,3-Dihydroxy-2-Methylxanthone	OH	CH_3	OH	H	H

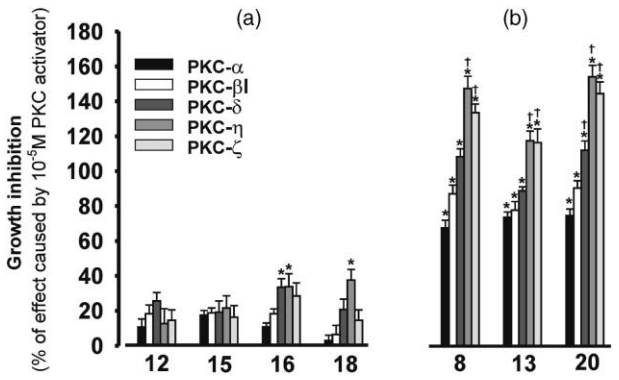


Figure 2. Effects of xanthones on the growth of yeast expressing individual PKC isoforms (α, βI, δ, η or ζ): (a) not effective xanthones; (b), xanthones effective in all PKC isoforms. Yeast cells expressing the indicated mammalian PKC isoform were incubated with xanthones (10^{-5} M) or solvent (DMSO, 0.1% final concentration; see Experimental for details). Shown are means ± SEM of 30–48 determinations. Yeast growth significantly different from solvent: $^{*}p < 0.05$ (paired Student's *t*-test), $^{\dagger}p < 0.05$ significantly higher than the maximal effect caused by 10^{-5} M of PKC activator (unpaired Student's *t*-test).

In the absence of galactose, neither PMA nor arachidonic acid altered yeast growth. Furthermore, 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).

To investigate whether xanthone derivatives (Table 1) could behave as PKC activators, yeast cells expressing PKC-α, PKC-βI, PKC-δ, PKC-η or PKC-ζ were incubated in the presence of each of these compounds. On a first approach each xanthone was tested in a single concentration (10^{-5} M). In the absence of galactose, none of the xanthones tested altered yeast growth (data not shown). In the presence of galactose, the majority of these compounds were able to inhibit the growth of yeast expressing individual PKC isoforms.

The series of 20 xanthone derivatives tested in the present study were first grouped according to their efficacy. Efficacy was evaluated by their ability to elicit a significant growth inhibition, compared to growth in the presence of solvent (DMSO; 0% growth inhibition) and growth in the presence of 10^{-5} M of the respective PKC acti-

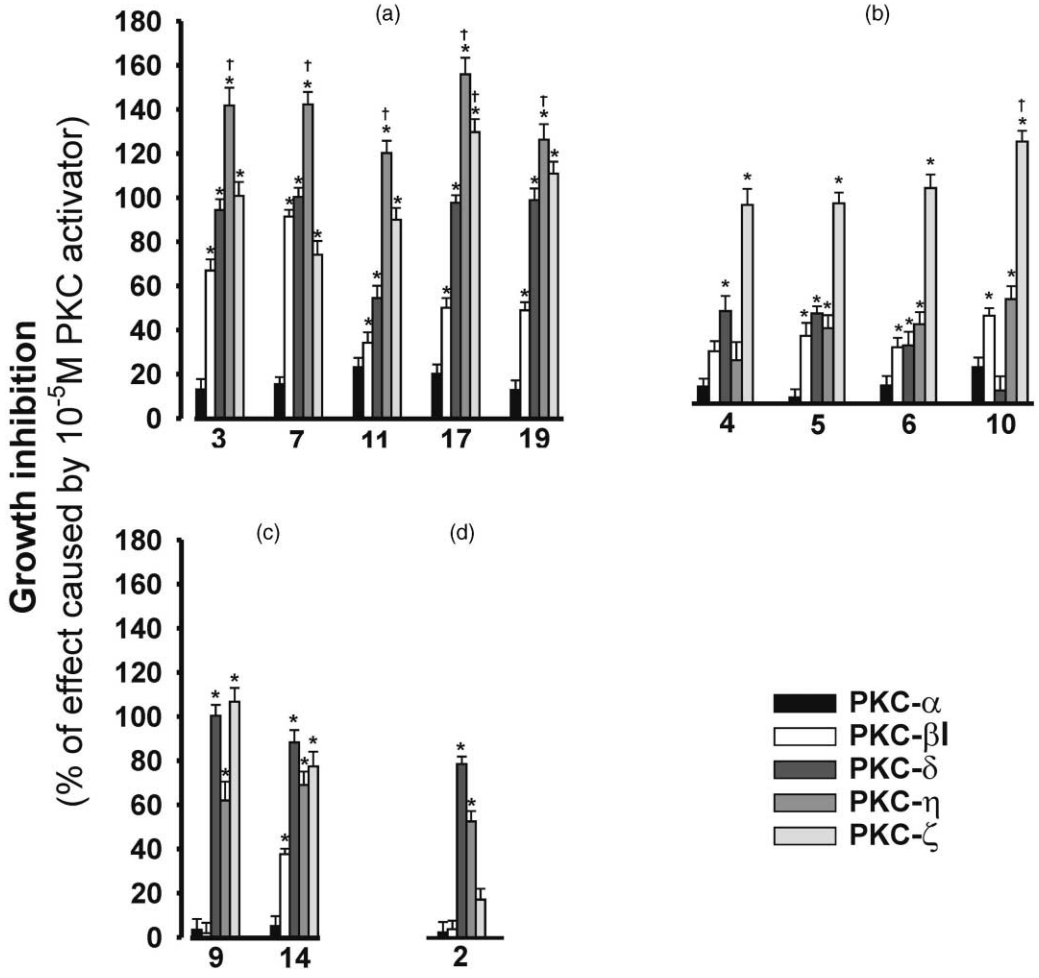


Figure 3. Effects of xanthones showed to be not effective on PKC-α, on the growth of yeast expressing individual PKC isoforms (α, βI, δ, η or ζ): (a) xanthones highly effective on PKC-η; (b) xanthones highly effective on PKC-ζ; (c) xanthones effective on nPKC and aPKC isoforms; (d) xanthones only effective on nPKC isoforms. Yeast cells expressing the indicated mammalian PKC isoform were incubated with xanthones (10^{-5} M) or solvent (DMSO, 0.1% final concentration; see Experimental for details). Shown are means ± SEM of 30–48 determinations. Yeast growth significantly different from solvent: $^{*}p < 0.05$ (paired Student's *t*-test), $^{\dagger}p < 0.05$ significantly higher than the maximal effect caused by 10^{-5} M of PKC activator (unpaired Student's *t*-test).

vator (PMA for PKC-α, -βI, -δ and -η; arachidonic acid for PKC-ζ; 100% growth inhibition). From the series of xanthones tested, a group of four (**12**, **15**, **16** and **18**) was considered not effective in any PKC isoform (Fig. 2a) because they did not cause a growth inhibition that reached at least half of the growth inhibition caused by the respective PKC activator.

A group of three xanthones (**8**, **13** and **20**) was effective in all PKC isoforms (Fig. 2b). It is important to highlight the efficacy of these xanthones on yeast expressing nPKCs (δ and η) and aPKC (PKC-ζ) isoforms. With the exception of xanthone **13** on yeast expressing PKC-δ, all caused inhibition of yeast growth that exceeded those caused by the standard PKC activator. On cPKCs the efficacy of these xanthones was lower than that caused by the standard PKC activator.

The remaining xanthones were effective in some but not in all PKC isoforms. They were tentatively grouped according to the isoform(s) in which they were not effective. A group of 12 xanthones was found to be inactive on PKC-α (Fig. 3). From this group, compounds **3**, **7**, **11**, **17**, and **19** (Fig. 3a) presented high efficacies on PKC-η, causing maximal inhibitions higher than those caused by PMA. Compounds **4**, **5**, **6** and **10** (Fig. 3b) caused a growth inhibition on yeast expressing PKC-ζ much more pronounced than that observed on yeast expressing the other isoforms. Compounds **9** and **14** were similarly effective on yeast expressing nPKCs and aPKC isoforms (Fig. 3c). Compound **2** was effective only on yeast expressing nPKCs isoforms (Fig. 3d).

Compound **1** presented a high efficacy on PKC-η, causing a maximal inhibition higher than that caused by 10^{-5} M PMA (Fig. 4), but differs from the group of compounds in Figure 3a because it was also effective on yeast expressing PKC-α.

The effective compounds were grouped, afterwards, according to their potency by comparing their IC_{50} values, that is the concentration of the xanthone that caused half of the growth inhibition caused by the standard PKC activator PMA (10^{-5} M; arachidonic acid for PKC-ζ). For this purpose, concentration-response curves were obtained and xanthones tested in the concentration range of 10^{-11}–10^{-5} M (see Fig. 5 for typical concentration–response curves obtained for xanthone **20**).

Compounds **8**, **13** and **20**, although effective in all isoforms tested, presented marked differences on their potency (see Table 2). In general they presented an higher potency on yeast expressing PKC-η. Compound **20** presented also a comparable high potency on yeast expressing PKC-ζ. They have also in common a low potency on yeast expressing cPKC isoforms, with the exception of compound **13** on yeast expressing PKC-βI. According to the IC_{50} values, the rank order of potency was, for compound **8**, PKC-η > -δ = -ζ > -α > -βI; for compound **13**, PKC-η > -ζ = -βI > -δ > -α and for compound **20**, PKC-η = -ζ > -δ > -βI > -α (for definition of the rank order of potency, the signal > was applied only when the IC_{50} of the compound placed at the left was significantly lower than that of the IC_{50} placed at the

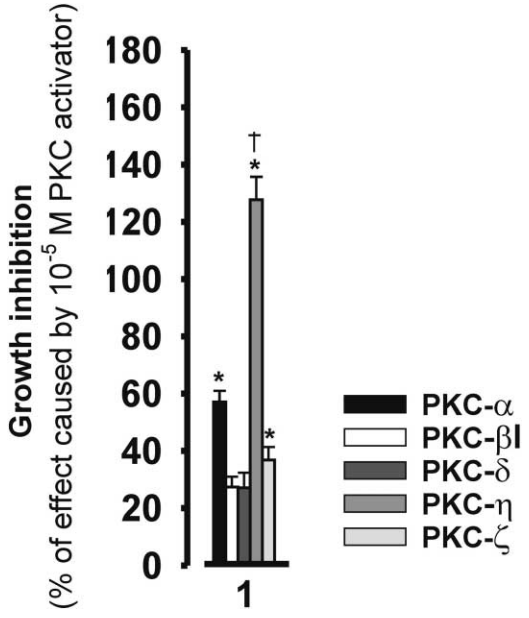


Figure 4. Effects of a xanthone showed to be effective on PKC-α and highly effective on PKC-η, on the growth of yeast expressing individual PKC isoforms (α, βI, δ, η or ζ). Yeast cells expressing the indicated mammalian PKC isoform were incubated with xanthone **1** (10^{-5} M) or solvent (DMSO, 0.1% final concentration; see Experimental for details). Shown are means ± SEM of 24–42 determinations. Yeast growth significantly different from solvent: $^{*}p < 0.05$ (paired Student's *t*-test), $^{\dagger}p < 0.05$ significantly higher than the maximal effect caused by 10^{-5} M of PKC activator (unpaired Student's *t*-test).

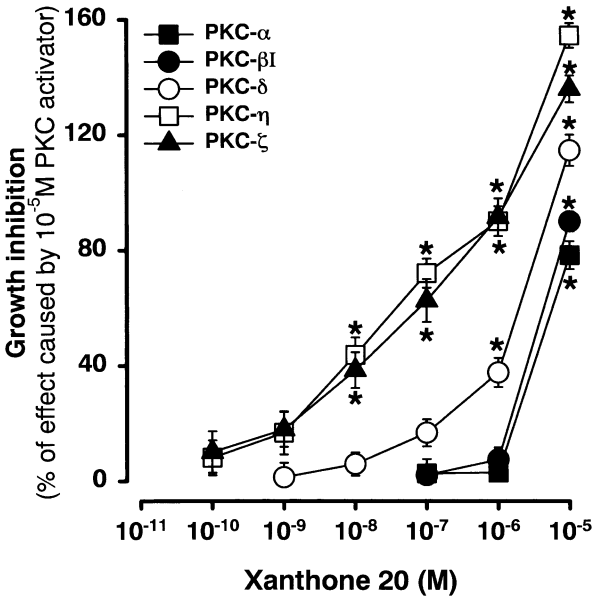


Figure 5. Typical concentration–response curves for the effects of a xanthone (**20**) on the growth of yeast expressing the indicated mammalian PKC isoforms (α, βI, δ, η or ζ). Results are expressed as % of the maximal effect caused by 10^{-5} M of PKC activator (100% growth inhibition was considered in each isoform that caused by 10^{-5} M of PKC activator). Shown are means ± SEM of 20–32 determinations. Yeast growth significantly different from solvent: $^{*}p < 0.05$ (paired Student's *t*-test).

Table 2. IC_{50} values of xanthone derivatives at the mammalian PKC-α, -βI, -δ, -η or -ζ

Compound	IC_{50} (nM)				
	PKC-α	PKC-βI	PKC-δ	PKC-η	PKC-ζ
1	6300±812**	*ND*	*ND*	7±2	*ND*
2	*ND*	*ND*	2510±630	9579±2055*	*ND*
3	*ND*	3981±744**	50±18**	2±0.7	13±2**
4	*ND*	*ND*	*ND*	*ND*	626±73
5	*ND*	*ND*	*ND*	*ND*	188±41
6	*ND*	*ND*	*ND*	*ND*	16±4
7	*ND*	101±12*	19±2	25±8	2000±986*
8	2512±367**	5000±507**	141±14**	9±2	151±21**
9	*ND*	*ND*	1032±190†	8700±916†	6±2
10	*ND*	*ND*	*ND*	*ND*	501±37
11	*ND*	*ND*	*ND*	3±1	2460±690**
13	2500±577**	126±34**	248±27**	1±0.8	79±16**
14	*ND*	*ND*	5±2	2876±1143*	2522±1090*
17	*ND*	9897±3580†	2410±414†	188±62†	4±0.8
19	*ND*	*ND*	1021±232**	2±1	21±10
20	5623±681**,†	3660±352**,†	1585±127**,†	16±4	28±10

IC_{50} values were considered to be the concentration that caused 50% growth inhibition (assuming that 100% growth inhibition was that caused by 10^{-5} M PMA for PKC -α, -βI, -δ and -η or by 10^{-5} M arachidonic acid for PKC-ζ). Xanthones were tested in a concentration range of 10^{-11}–10^{-5} M. Shown are means±SEM of 20–32 determinations. *ND*; non determinable (when the maximal response reached was lower than 50% growth inhibition). Significant differences: relatively to PKC-δ, $*p < 0.05$; relatively to PKC-η, $**p < 0.05$; relatively to PKC-ζ, $^{\dagger}p < 0.05$ (unpaired Student's *t*-test).

right of the signal; otherwise = was applied; $p < 0.05$; unpaired Student's *t*-test).

Compounds **3**, **7**, **11** and **17** were all inactive on yeast expressing PKC-α and presented high potencies on yeast expressing PKC-η. Compound **7** presented also a comparable high potency on yeast expressing PKC-δ whereas compounds **3** and **17** also presented high potencies on yeast expressing PKC-ζ (see Table 2). According to the IC_{50} values, the rank order of potency obtained was, for compound **3**, PKC-η > -ζ > -δ > -βI; for compound **7**, PKC-η = -δ > -βI > -ζ, for compound **11**, PKC-η > -ζ and for compound **17**, PKC-ζ > -η > -δ > -βI.

Compounds **1** and **11** presented a remarkable potency and selectivity towards PKC-η (see Table 2). Compound **1** presented an IC_{50} value 900 times lower than that for the other isoform in which it was also active (PKC-α). Similarly, compound **11** presented an IC_{50} value 820 times lower than that for the other isoform in which it was active (PKC-ζ).

Compounds **4**, **5**, **6** and **10** presented a high selectivity towards PKC-ζ. For these xanthones IC_{50} values were determinable only on PKC-ζ. Nevertheless, they differ on their potency, being compound **6** the most potent followed by **5** and then by compounds **4** and **10** (see Table 2).

Compounds **9**, **14** and **19** have IC_{50} values determinable only on yeast expressing isoforms of the nPKC and aPKC families (see Table 2). Interestingly, each xanthone showed to be particularly potent on a different PKC isoform: **9** on PKC-ζ; **14** on PKC-δ and **19** on PKC-η.

Compound **2** was active only on yeast expressing nPKC isoforms, with a potency higher on yeast expressing PKC-δ than on yeast expressing PKC-η (see Table 2). However, the IC_{50} values were much higher (in the micromolar range) than those presented by xanthones referred as selective to nPKC isoforms.

Discussion

The yeast phenotypic assay, which uses transformed yeast expressing individual mammalian PKC isoforms, has been proposed as an alternative rapid and simple experimental assay for the study of PKC-activating potential of drug candidates. In the present study the mammalian PKC-α, -βI, -δ, -η or -ζ isoforms were expressed in the same yeast strain. After exposure to the PKC activator (PMA or arachidonic acid), a concentration-dependent growth inhibition was detected in yeast expressing each of the PKC isoforms tested. Since the PKC activator-induced growth inhibition was observed only when there was expression of a mammalian PKC isoform (as confirmed by immunoblotting) it is assumed that, under the present experimental conditions, the drug-induced growth inhibition reflects activation of the expressed mammalian PKC isoform.

The majority of the xanthones tested revealed effects compatible with activation of PKC isoforms of the cPKC, nPKC and aPKC families, although, in general, they showed a very low activity on isoforms of the cPKC family, particularly on PKC-α: among the 20 xanthones tested only four (**1**, **8**, **13** and **20**) were able to activate this isoform.

PKC activators like phorbol esters and arachidonic acid, activate PKC by interacting at the regulatory domain[23] but by a distinct mechanism.[24] To activate

PKC, phorbol esters seem to require two cysteine-rich zinc-fingers at the C1 region of the regulatory domain (C1a and C1b).[4] Atypical PKC isoforms lack the second cysteine-rich motif, what explains the inactivity of phorbol esters on isoforms of this family. Arachidonic acid activates PKC by a mechanism that does not require the repeated zinc finger motifs and, thus, arachidonic acid acts as an universal PKC activator.[25] Xanthones activated isoforms of all PKC families, including isoforms of the aPKC family, suggesting that xanthones interact with PKC by a mechanism similar to that of arachidonic acid.

The xanthones tested showed marked activities on nPKC and aPKC. Some were more potent than the standard PKC activator and presented a remarkable selectivity for a specific isoform. These simple xanthones were especially active on PKC-η and on PKC-ζ, in which only four compounds (**4**, **5**, **6** and **10**) and two compounds (**1** and **2**) were inactive, respectively.

Efforts have been made in the past by several groups to study, by using molecular modeling approaches, structure–activity relationships and to establish a common pharmacophore shared by structurally different PKC-activators, despite the lack of 3-D-structural data for individual PKC isoforms.[26] Our study shows that small structural changes on the xanthone framework can lead to dramatic alterations of its activity/selectivity towards individual PKC isoforms. Furthermore, it revealed some insights about the structural requirements of xanthones for PKC activation. Positions 3 and 5 do not seem to be recommendable for introduction of hydroxy/methoxy substituents, once xanthones **15**, **16** and **18** were not effective in any PKC isoform studied. On the other hand, the methoxylation of positions 2 and 3 were shown to be the most favoured substitution to achieve activity in all PKC isoforms tested (compound **13** compared with compound **12**).

The present study revealed new compounds which showed high selectivity for individual PKC isoforms: compound **14** for PKC-δ and compounds **4**, **5**, **6**, **9** and **10** for PKC-ζ, isoforms reported to be of crucial importance in the apoptotic process;[3] and compounds **1** and **11** for PKC-η, an isoform suggested to be involved in growth, differentiation and carcinogenesis of epithelial tissues.[27] Therefore xanthones can be useful for a detailed study of the physiological and pathophysiological roles of PKC isoforms.

Conclusions

This study shows that simple xanthones can interact with mammalian PKC isoforms of the cPKC, nPKC and aPKC families, causing an effect compatible with PKC activation. The high potency and selectivity presented suggests that xanthones can become an important group to look for potent and isoform-selective PKC activators which can be useful to elucidate the physiological and pathophysiological roles of individual PKC isoforms.

Experimental

Chemistry

Purifications of compounds were performed by column chromatography using Merck silica gel 60 (0.50–0.20 mm), flash chromatography using Merck silica gel 60 (0.040–0.063 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 and DRX 300 instruments. 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.

Xanthone (**1**) was purchased from Sigma Aldrich (Sintra, Portugal). The following materials were synthesized and purified by the described procedures. The synthesis of the following compounds have already been described:[28] 1-hydroxyxanthone (**2**), 1-methoxyxanthone (**3**), 2-methoxyxanthone (**5**), 2-hydroxyxanthone (**4**), 3-hydroxyxanthone (**6**), 3-methoxyxanthone (**7**), 4-hydroxyxanthone (**8**), 4-methoxyxanthone (**9**), 3,5-dihydroxyxanthone (**15**), 3,5-dimethoxyxanthone (**16**), 3-hydroxy-5-methoxyxanthone (**18**), 3,4-dimethoxyxanthone (**14**), 3-hydroxy-4-methoxyxanthone (**17**) and 4-hydroxy-3-methoxyxanthone (**19**).

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

1,2-Dihydroxyxanthone (10). Obtained by demethylation of **11** according to the procedure described.[29,30] Mp 163–165 °C, methylene chloride/hexane,[31] 166–167 °C, ethanol aq); $\upsilon_{máx}$ (cm^{-1}) KBr: 3467, 1606, 1475, 1365, 1288, 1045, 748; ^1H NMR (DMSO-d_6, 300.13 MHz) δ: 12.45 (1-O*H*), 9.42 (2-O*H*), 8.16 (1H, dd, $J=7.9$ and 1.6 Hz, H-8), 7.81 (1H, ddd, $J=8.4$, 7.0 and 1.6 Hz, H-6), 7.60 (1H, dd, $J=8.4$ and 0.8 Hz, H-5) 7.46 (1H, ddd, $J=7.9$, 7.0 and 0.8 Hz, H-7), 7.32 (1H, d, $J=9.0$ Hz, H-4), 6.96 (1H, d, $J=9.0$ Hz, H-3); ^{13}C NMR (DMSO-d_6, 75.47 MHz) δ: 182.3 (C-9), 155.9 (C-4b), 148.3 (C-4a), 147.6 (C-1), 140.2 (C-2), 136.4 (C-6), 125.4 (C-8), 124.5 (C-3), 124.2 (C-7), 119.3 (C-8a), 118.0 (C-5), 108.8 (C-9a), 106.2 (C-4); MS *m*/*z* (rel int): 230 (1, $[M+2]^{+\cdot}$), 229 (1, $[M+1]^{+\cdot}$), 228 (70, $[M^{+\cdot}]$), 199 (13), 126 (5), 115 (4), 78 (95), 63 (100).

1,2-Dimethoxyxanthone (11).[32] Mp 133–135 °C, methylene chloride/hexane,[31] 130 °C; $\upsilon_{máx}$ (cm^{-1}) KBr: 3488, 3436, 3377, 1662, 1461, 1292, 1085, 1049, 742; ^1H NMR (DMSO-d_6, 200.13 MHz) δ: 8.12 (1H, dd, $J=7.9$ and 1.7 Hz, H-8), 7.81 (1H, ddd, $J=7.6$, 7.4 and 1.7 Hz, H-6), 7.63 (1H, d, $J=9.3$ Hz, H-4), 7.56 (1H, d, $J=7.4$ Hz, H-5), 7.44 (1H, dd, $J=7.9$, 7.6 and 1.1 Hz, H-7), 7.40 (1H, d, $J=9.3$ Hz, H-3), 3.86 (3H, s, 1-OCH_3), 3.80 (3H, s, 2-OCH_3); ^{13}C NMR (DMSO-d_6, 50.03 MHz) δ: 175.2 (C-9), 154.7 (C-4b), 150.3 (C-2), 148.9 (C-1), 147.5 (C-4a), 135.0 (C-6), 126.0 (C-8), 123.9 (C-7), 121.4 (C-8a), 120.7 (C-5), 117.6 (C-3), 116.4 (C-9a), 113.2 (C-4), 61.0

(1-OCH$_3$), 56.6 (2-OCH$_3$); MS *m*/*z* (rel int): 258 (3, $[M+2]^{+\cdot}$), 257 (25, $[M+1]^{+\cdot}$), 256 (100, $[M^{+\cdot}]$), 241 (83), 227 (45), 223 (18), 213 (59), 196 (14), 184 (10), 170 (11), 155 (13), 139 (15), 127 (13), 121 (18), 114 (12), 104 (6), 92 (11), 84 (5), 77 (17), 63 (12).

2,3-Dihydroxyxanthone (12). Obtained by demethylation of **14** according to the procedure previously described.[30] Mp >330 °C, acetone/hexane[30] >350 °C ethyl acetate,[33] 293–295 °C chloroform/methanol); $\upsilon_{máx}$ (cm^{-1}) KBr: 3400, 1624, 1464, 1311, 1290, 1145, 746; ^{1}H NMR (DMSO-d_6, 300.13 MHz) δ: 8.12 (1H, dd, *J* = 7.5 and 1.6 Hz, H-8), 7.78 (1H, ddd, *J* = 8.4, 6.8 and 1.7 Hz, H-6), 7.58 (1H, d, *J* = 8.4 Hz, H-5), 7.43 (1H, s, H-1), 7.42 (1H, ddd, *J* = 7.5, 6.8 and 1.9 Hz, H-7), 6.92 (1H, s, H-4); ^{13}C NMR (DMSO-d_6, 75.47 MHz) δ: 175.0 (C-9), 155.7 (C-4b), 154.2 (C-4a), 151.3 (C-3), 144.1 (C-2), 134.7 (C-6), 125.9 (C-8), 124.1 (C-7), 120.9 (C-8a), 118.1 (C-5), 113.7 (C-9a), 108.9 (C-1), 103.0 (C-4); MS *m*/*z* (rel int): 230 (0.34, $[M+2]^{+\cdot}$), 229 (2.4, $[M+1]^{+\cdot}$), 228 (16, $[M^{+\cdot}]$), 200 (0.96), 171 (0.80), 126 (1.8), 115 (0.96), 100 (8), 78 (86), 63 (100).

2,3-Dimethoxyxanthone (13). The 2-(3′,4′-dimethoxyphenoxy)benzoic acid obtained initially by an Ullman reaction[34] underwent an appropriate cyclization to **13** according to the procedure previously described[35,36] Mp 170–171 °C, methylene chloride/pentane[36] 164–165 °C, acetone,[33] 157–158 °C, chloroform); $\upsilon_{máx}$ (cm^{-1}) KBr: 3500, 3440, 3407, 1649, 1466, 1313, 1234, 1005, 762; ^{1}H NMR (DMSO-d_6, 200.13 MHz) δ: 8.16 (1H, dd, *J* = 7.9 and 1.5 Hz, H-8), 7.82 (1H, ddd, *J* = 8.4, 7.4 and 1.5 Hz, H-6), 7.60 (1H, d, *J* = 8.4 Hz, H-5), 7.49 (1H, s, H-1), 7.44 (1H, ddd, *J* = 7.9, 7.4, and 0.9 Hz, H-7), 7.20 (1H, s, H-4), 3.94 (3H, s, 3-OC*H*$_3$), 3.87 (3H, s, 2-OC*H*$_3$); ^{13}C NMR (DMSO-d_6, 50.03 MHz) δ: 174.8 (C-9), 155.7 (C-4b), 155.6 (C-4a), 152.0 (C-3), 146.7 (C-2), 134.8 (C-6), 125.9 (C-8), 124.3 (C-7), 120.9 (C-8a), 118.0 (C-5), 114.0 (C-9a), 104.7 (C-1), 100.5 (C-4), 56.6 (3-OCH$_3$), 55.9 (2-OCH$_3$); MS *m*/*z* (rel int): 258 (1, $[M+2]^{+\cdot}$), 257 (8, $[M+1]^{+\cdot}$), 256 (40, $[M^{+\cdot}]$), 241 (11), 185 (3), 170 (3), 142 (3), 78 (89), 63 (100).

1,3-Dihydroxy-2-methylxanthone (20). Obtained according to the procedure previously described.[37,38]

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, 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.[39] 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.[6] Detergent lysates were separated on 10% SDS-polyacrylamide gels (Mini-Protean II, BioRad, Hercules, CA, USA). Proteins were electrophoretically transferred to nitrocellulose membranes and probed on immunoblots with specific rabbit antibodies to either PKC-α, PKC-βI, PKC-δ, PKC-η or PKC-ζ and with a secondary alkaline phosphatase-conjugated anti-rabbit IgG. Standard recombinants of 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) were used as positive controls.

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-well 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 when it was reached a 'steady-state growth inhibition' (period of time during which maximal inhibition of growth was reached and remained constant or changed only slightly). 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, arachidonic acid or xanthones). 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 standardise 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 xanthones were expressed as percentage of that effect. For determination of concentration–response curves, each drug was tested in concentrations ranging from 10^{-11} to 10^{-5} M and the concentration of drug that caused half of the 100% growth inhibition (IC_{50}) was calculated.

Statistical analysis. Results are given as arithmetic means ± SEM and n represents the number of determinations. Differences between means were tested for significance using the paired or unpaired Student's t-test, as indicated in the figure legends. A p value lower than 0.05 was taken to be statistically significant.

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