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Isoform-selectivity of PKC Inhibitors Acting at the Regulatory and Catalytic Domain of Mammalian PKC-α, -βI, -δ, -η and -ζ

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The aim of the present study was to compare the potency of a series of widely used PKC inhibitors acting either at the regulatory (NPC 15437, tamoxifen and D-sphingosine) or at the catalytic domain (Ro 32-0432, chelerythrine and rottlerin) on individual mammalian PKC isoforms of the classical (α and βI), novel (δ and η) and atypical (ζ) PKC families, using the yeast phenotypic assay, in order to determine their isoform-selectivity. The PKC inhibitors studied presented differences in their ability to reduce the effect of the appropriate PKC activator (estimated as EC_{50} ratios) which was interpreted as an index of PKC inhibitory potency. In general, the more marked inhibition was observed on novel PKC isoforms, particularly on PKC-η. This study indicates promising isoform-selectivity of some PKC inhibitors, namely NPC 15437 for PKC-η or rottlerin for both novel PKC isoforms. It also suggests that the PKC domain involved in the inhibition does not seem to be relevant for the potency and isoform-selectivity of PKC inhibitors.

Keywords: Protein kinase C; PKC isoforms; PKC inhibitors; Yeast phenotypic assay

INTRODUCTION

Protein kinase C (PKC) is a family of serine–threonine kinases that comprises at least 13 isoforms, classified into three groups: the classical PKCs (cPKCs), which include isoforms α, βI, βII and γ; the novel PKCs (nPKCs), which include isoforms δ, ε, η and θ and the atypical PKCs (aPKCs), which include isoforms ζ and ι/λ.[1,2] Recently, a new member of the PKC family, PKCμ/PKD, has been reported, but its inclusion in this family remains controversial.[1]

The general structure of a PKC molecule consists of a regulatory and a catalytic domain, found at the —NH_2 and —COOH terminus respectively, containing conserved (C1–C4) regions of extended sequence homology as well as variable (V1–V5) regions.[2,3]

PKC isoforms are involved in transmembrane signal transduction pathways,[3,4] regulating a wide variety of cellular functions such as growth, differentiation, tumor promotion and apoptosis.[1,2,4] Individual PKC isoforms differ in their expression patterns and substrate specificities, strongly suggesting that each isoform may be involved in distinct regulatory processes within the cell.[5] Activation of PKC isoforms occurs in a number of pathological states, such as chronic inflammation and proliferative diseases, which emphasises the therapeutic potential of isoform-selective PKC inhibitors.[2,4,6–8] Furthermore, availability of isoform-selective PKC inhibitors may also provide important pharmacological agents to better define the physiological and pathophysiological functions of each PKC isoform.[4,8,9]

A large variety of structurally distinct PKC inhibitors have been identified or developed so far,[7] namely chelerythrine,[10] NPC 15437,[11–13] Ro 32-0432,[2,9,14,15] tamoxifen,[16–20] rottlerin[9,21] and D-sphingosine.[2,22,23] They differ in their site of interaction with PKC: chelerythrine,[8,10] Ro 32-0432[2,8,14,15] and rottlerin[8,9,21] interact with PKC at the catalytic domain whereas NPC 15437,[11–13] tamoxifen[8,16,17] and D-sphingosine[2,8,22,23] interact at the regulatory domain.

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Although these compounds have been widely used as PKC inhibitors, little information is available about their isoform-selectivity. Study of isoform-selectivity has been hampered by methodological limitations,[24] such as the coexistence of several PKC 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 which uses transformed yeast expressing a single functional mammalian PKC isoform. This assay is based on the inhibition of yeast growth (reflecting an increase in the cell doubling time) caused by PKC activators that is proportional to the degree of PKC activation.[25,26] In this assay, PKC inhibitors reduce growth inhibition caused by an activator[24,27] and comparison of that reduction may be taken as an index of potency of a given PKC inhibitor against the expressed isoform.

Because only a single mammalian PKC isoform is expressed, this method makes possible the comparison of the potency of a compound on different isoforms, under similar experimental conditions. Since expression of the mammalian PKC is dependent on the presence of a transcription inducer (galactose), effects in the absence of galactose (no expression) allows detection of effects non-mediated by the expressed PKC isoform.

In the present study, effects of the PKC inhibitors chelerythrine, NPC 15437, Ro 32-0432, tamoxifen, rottlerin and D-sphingosine on PKC isoforms α and βI (cPKCs), δ and η (nPKCs) and ζ (aPKCs) were compared using the yeast phenotypic assay. Our results indicate that these PKC inhibitors may present some isoform-selectivity and that the yeast phenotypic assay may be an appropriate method to investigate the isoform-selectivity of putative PKC inhibitors.

MATERIALS AND METHODS

Chemicals

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 chloride, leupeptin, NPC 15437 dihydrochloride (*R*-2,6-diamino-*N*-[[1-(1-oxotridecyl)-2-piperidinyl]methyl]-hexanamide dihydrochloride), pepstatin A, phenylmethylsulfonyl fluoride, PMA (phorbol 12-myristate 13-acetate), Ro 32-0432 (2-(8-[(dimethylamino)methyl]-6,7,8,9-tetrahydropyrido[1,2-a]indol-3-yl)-3-(1-methylindol-3-yl)maleimide, hydrochloride), rottlerin (mallotoxin), D-sphingosine (*trans*-D-*erythro*-sphingosine) were from Sigma Aldrich (Sintra, Portugal). Tamoxifen citrate was from Tocris (Bristol, UK). 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 provided 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 provided 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.[28] 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 in the medium to induce transcription of the mammalian PKC gene.

Cell Lysis and Immunoblotting

Cell lysis was performed basically as described.[25] The protein concentration was determined using the *Coomassie® Protein Assay Reagent Kit*, (Pierce, Biocontec, Lisboa, 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 for the individual

mammalian PKC isoforms and revealed with a secondary alkaline phosphatase-conjugated anti-rabbit IgG (AP-10).

Yeast Phenotypic Assay

Transformed yeast cultures were incubated in leucine-free medium. Optical density measurements 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 1.0, 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 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" was reached (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 the activators used (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 a 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 attainable on different isoforms; 0% growth inhibition would occur when yeast growth in the presence of a drug was identical to that in the presence of solvent.

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

Statistical Analysis

Results are given as arithmetic means ± SEM of *n* 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 considered to denote a statistically significant difference.

RESULTS

Expression of mammalian PKC-α, -βI, -δ, -η or -ζ was confirmed by immunoblotting, using protein extracts of yeast cells, transformed with a plasmid containing the gene for one of the mammalian PKC isoforms, grown in the presence of the transcription inducer (2% galactose). Expression of the PKC isoform 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 (see Figure 1).

PMA (considered as the standard activator for the classical and novel PKC isoforms) was tested on yeast expressing PKC-α, -βI, -δ or -η, in concentrations up to 10^{-5} M (higher concentrations could not be tested due to its low solubility in the culture medium). Since atypical PKC isoforms are not activated by phorbol esters,[29] arachidonic acid (up to 10^{-5} M) was used as the activator for PKC-ζ.[30]

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

Maximal values of growth inhibition caused by 10^{-5} M of the appropriate PKC activator (PMA for PKC-α, -βI, -δ and -η or arachidonic acid for PKC-ζ), on the PKC isoforms tested, are presented in Table 1 and were assumed to correspond to the maximal growth inhibition attainable by PKC activation for the isoform expressed. Effects of lower concentrations of PKC activator were expressed as a percentage of that maximal growth inhibition.

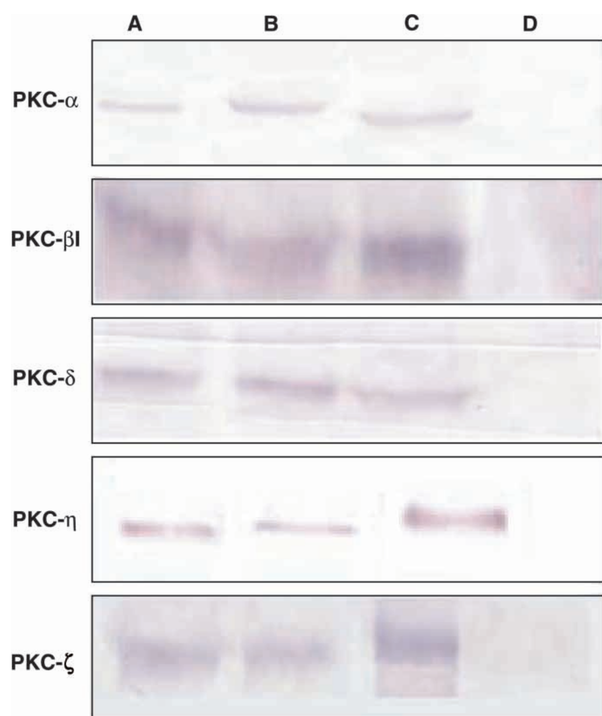

FIGURE 1 Immunodetection of PKC-α, -βI, -δ, -η and -ζ isoforms expressed in transformed *Saccharomyces cerevisiae* (CG379). Individual immunoblots are presented in a horizontal arrangement and were obtained from protein extracts (~ 40 μg protein/lane) from cultures grown in selective medium with 2% galactose (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).

In the presence of galactose, the appropriate PKC activator caused a concentration-dependent inhibition of the growth of yeast expressing the mammalian PKC isoform. For PMA, the EC_{50} values (nM) were (for the isoform indicated) 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) ($n = 64$). 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 ($n = 64$).

In the absence of galactose, chelerythrine, Ro 32-0432, tamoxifen and D-sphingosine, all tested at a concentration of 10^{-5} M, did not influence yeast growth. However, NPC 15437 (10^{-5} M) inhibited yeast growth to 73.0 ± 4.6% ($n = 32$), and rottlerin (10^{-5} M) increased yeast growth to 114.1 ± 10.1% ($n = 36$). At the concentration of 10^{-6} M neither NPC 15437 nor rottlerin influenced yeast growth. Therefore, 10^{-6} M was the maximal concentration of NPC 15437 and rottlerin used when expression of the mammalian PKC isoform was induced (galactose present in the medium). These maximal concentrations of PKC inhibitors that did not alter yeast growth, in the absence of mammalian PKC isoform expression, was defined as the maximal concentration feasible.

TABLE I Yeast growth inhibition caused by 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]

[a] Growth inhibition caused by 10^{-5} M arachidonic acid was 23.9 ± 0.7 ($n = 56$). 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.

In the yeast phenotypic assay, a PKC inhibitor reduces growth inhibition caused by a PKC activator.[24,27] However, a direct stimulation of yeast growth caused by the PKC inhibitor alone has also been reported.[24] Therefore, as a first approach, PKC inhibitors were tested alone and at the maximal concentration feasible: 10^{-5} M for chelerythrine, Ro 32-0432, tamoxifen and D-sphingosine; 10^{-6} M for NPC 15437 and rottlerin. As shown in Figure 2, PKC inhibitors stimulated growth of yeast expressing individual mammalian PKC isoforms; the only exception was D-sphingosine, which only slightly stimulated yeast growth. A reduction of the concentration reduced the growth stimulation. At 10^{-8} M, none of the PKC inhibitors stimulated yeast growth (Figure 2).

In a subsequent series of experiments, the ability of PKC inhibitors to reduce growth inhibition caused by PKC activators (PMA; arachidonic acid for PKC-ζ) was tested. PKC inhibitors were tested both at the maximal concentration feasible (at which they generally caused some growth stimulation) and at 10^{-8} M (concentration at which none caused a significant stimulation of yeast growth). Effects of chelerythrine, NPC 15437, Ro 32-0432, tamoxifen, rottlerin and D-sphingosine on the growth inhibition caused by the appropriate PKC activator are shown in Figures 3, 4, 5, 6, 7 and 8, respectively. At the maximal concentration feasible, chelerythrine, NPC 15437, Ro 32-0432, tamoxifen and rottlerin markedly reduced effects of the PKC activator, causing a shift to the right of the concentration-response curve to the PKC activator (compare open and filled circles of Figures 3, 4, 5, 6 and 7). Surprisingly, D-sphingosine caused a biphasic response on cPKCs and nPKCs: at 10^{-5} M increased the PMA induced growth inhibition whereas at 10^{-6} M already reduced the effect of PMA (compare open circles and filled triangles of Figure 8).

Chelerythrine, NPC 15437, Ro 32-0432, tamoxifen and rottlerin caused a shift to the right of the concentration-response curve for the PKC activator that, in general, was concentration-dependent (the displacement caused by 10^{-8} M was less

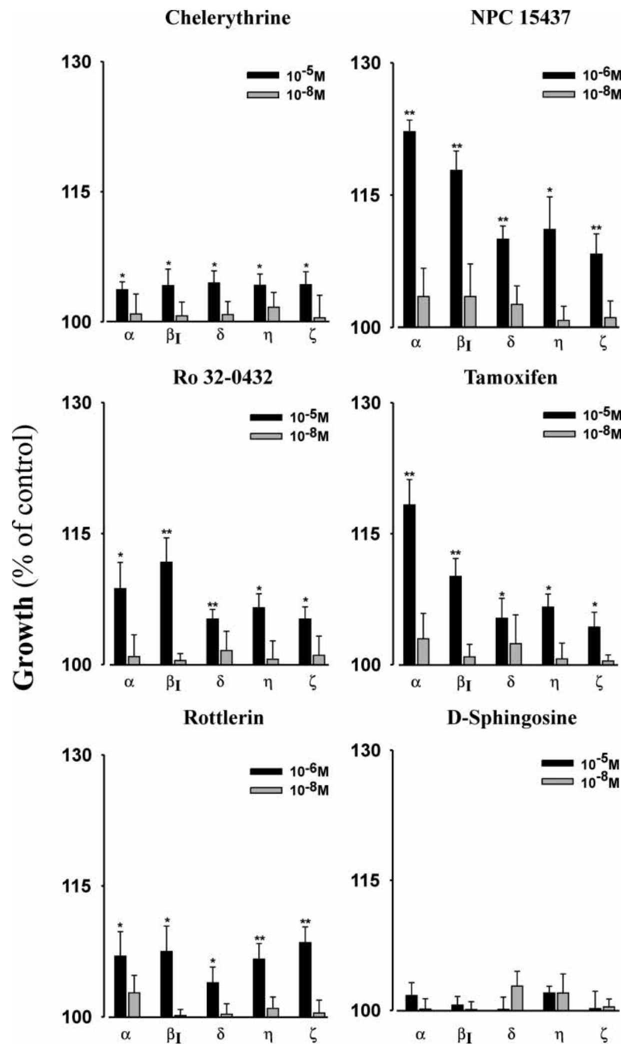

FIGURE 2 Effects of the indicated PKC inhibitor on the growth of yeast expressing individual PKC isoforms (α, βI, δ, η or ζ). Yeast cells expressing the indicated mammalian PKC isoform were incubated with the PKC inhibitor or solvent (DMSO; 0.1% final concentration). Results are expressed as % of growth. 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).

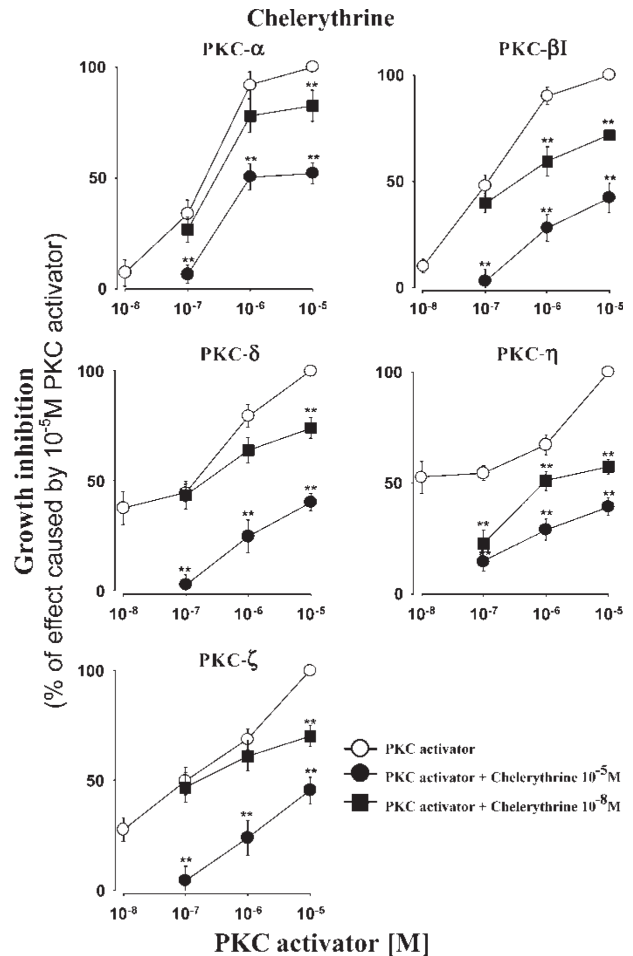

FIGURE 3 Interaction experiments for chelerythrine: concentration-response curves for PKC activator alone (open circles) and in the presence of 10^{-5} M (filled circles) or 10^{-8} M (filled squares) chelerythrine. Results are expressed as % of the maximal effect caused by 10^{-5} M PKC activator (PMA for PKC-α, -βI, -δ and -η; arachidonic acid for PKC-ζ). Shown are means ± SEM of 16–20 determinations. Significantly different from growth inhibition caused by PKC activator alone: $*P < 0.05$, $**P < 0.001$ (unpaired Student's t test).

pronounced than that caused by 10^{-5} M or 10^{-6} M; compare filled circles and squares of Figures 3, 4, 5, 6 and 7). However, in some cases (NPC 15437 on PKC-βI and -δ, Ro 32-0432 on PKC-η, tamoxifen on PKC-η and rottlerin on PKC-δ and -η), no concentration-dependence was found, the shift to the right being similar for the highest and the lowest concentration tested ($P > 0.05$; unpaired Student's t test).

To compare the potency of PKC inhibitors, ratios of EC_{50} values obtained in the presence and absence of each PKC inhibitor were calculated (EC_{50} is the concentration of the PKC activator that caused 50% of the growth inhibition caused by 10^{-5} M of the appropriate PKC activator). Only EC_{50} ratios obtained in the presence of 10^{-8} M of PKC inhibitor were used to estimate the PKC inhibitor potency on the various isoforms tested (Table 2). In general, PKC inhibitors showed the highest EC_{50} ratios on nPKC isoforms. The highest EC_{50} ratio was found for NPC 15437 on PKC-η, which was about 109 times higher than that obtained on the second highest ratio (observed on PKC-α). Chelerythrine, tamoxifen and Ro 32-0432 also caused their highest EC_{50} ratios on yeast expressing PKC-η but the differences between EC_{50} ratios obtained on PKC-η and on the isoform where the second highest EC_{50} ratio was observed (PKC-βI for chelerythrine; PKC-δ for tamoxifen and Ro 32-0432) were smaller than that observed for NPC 15437 (Table 2). Although rottlerin also caused significant EC_{50} ratios on PKC-η, it shared this high potency with PKC-δ upon which the EC_{50} ratio even tended to be higher (about 1.4 times) than that

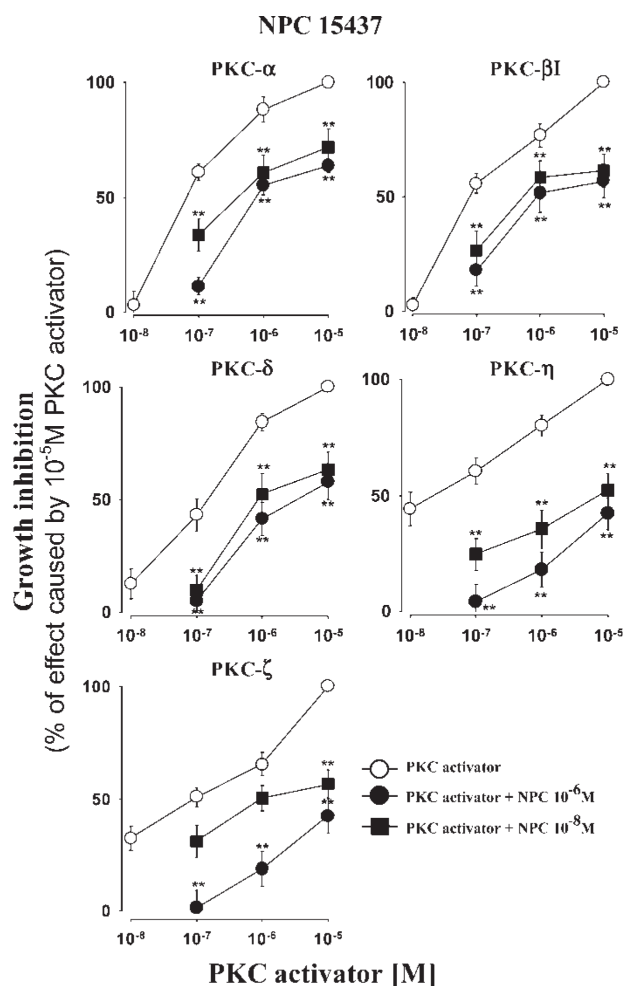

FIGURE 4 Interaction experiments for NPC 15437: concentration-response curves for PKC activator alone (open circles) and in the presence of 10^{-6} M (filled circles) or 10^{-8} M (filled squares) NPC 15437. Results are expressed as % of the maximal effect caused by 10^{-5} M PKC activator (PMA for PKC-α,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

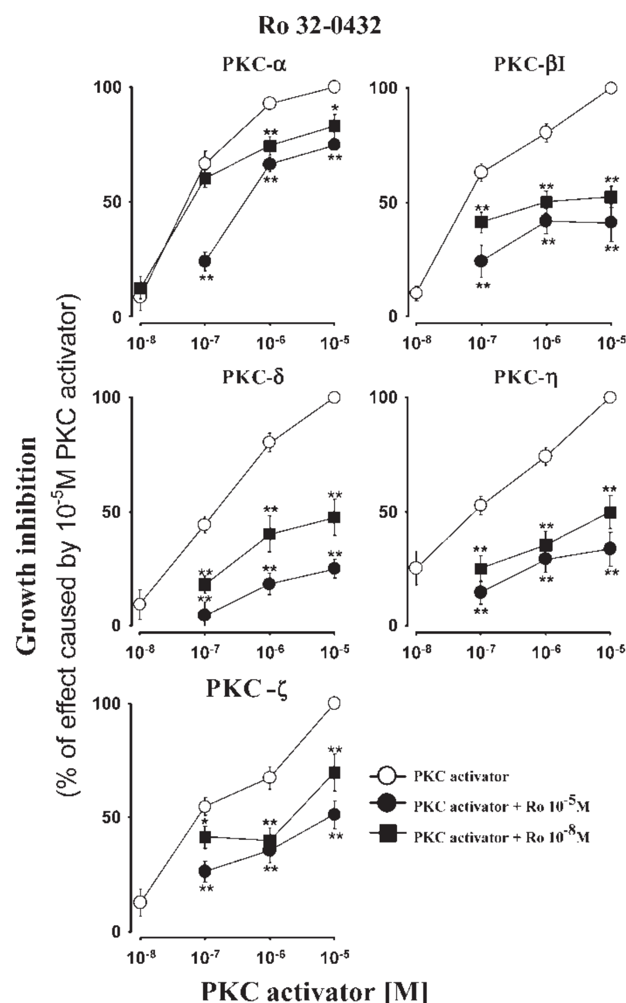

FIGURE 5 Interaction experiments for Ro 32-0432: concentration-response curves for PKC activator alone (open circles) and in the presence of 10^{-5} M (filled circles) or 10^{-8} M (filled squares) Ro 32-0432. Results are expressed as % of the maximal effect caused by 10^{-5} M PKC activator (PMA for PKC-α, -βI, -δ and -η; arachidonic acid for PKC-ζ). Shown are means ± SEM of 16–20 determinations. Significantly different from growth inhibition caused by PKC activator alone: $^*P < 0.05$, $^{**}P < 0.001$ (unpaired Student's t test).

obtained on PKC-η (Table 2). D-sphingosine was, in general, the PKC inhibitor with the lowest EC_{50} ratio value on all the isoforms tested.

According to the obtained EC_{50} ratios, the rank order of potency was, for chelerythrine, PKC-η > −βI > −α = −ζ = −δ; for NPC 15437, PKC-η > −α = −ζ > βI > −δ; for Ro 32-0432, PKC − η > −δ > −ζ = −βI > −α; for tamoxifen, PKC-η > −δ > −ζ = −βI; for rottlerin, PKC-δ = −η > −ζ > −βI and for D-sphingosine, PKC-δ > −η = −ζ = −α = −βI (for definition of the rank order of potency, the sign > was applied only when the EC_{50} ratio of the isoform placed on the left was significantly higher than that of the EC_{50} ratio placed on the right of the sign; otherwise = was applied; $P < 0.05$; unpaired Student's t test).

DISCUSSION

Despite the use of PKC inhibitors as pharmacological or therapeutic agents, little information is available concerning their isoform-selectivity, particularly in an eukaryotic environment[4,24] and on a system where a single mammalian PKC isoform is expressed. The present work is an attempt to characterise the isoform-selectivity of a group of widely used PKC inhibitors on a set of mammalian isoforms of the three main PKC families, expressed under identical experimental conditions and using the yeast phenotypic assay.

In the present study, mammalian PKC-α, -βI, -δ, -η or -ζ was functionally expressed in yeast cells of the same strain of *S. cerevisiae*. On the yeast phenotypic assay, PKC activation induces

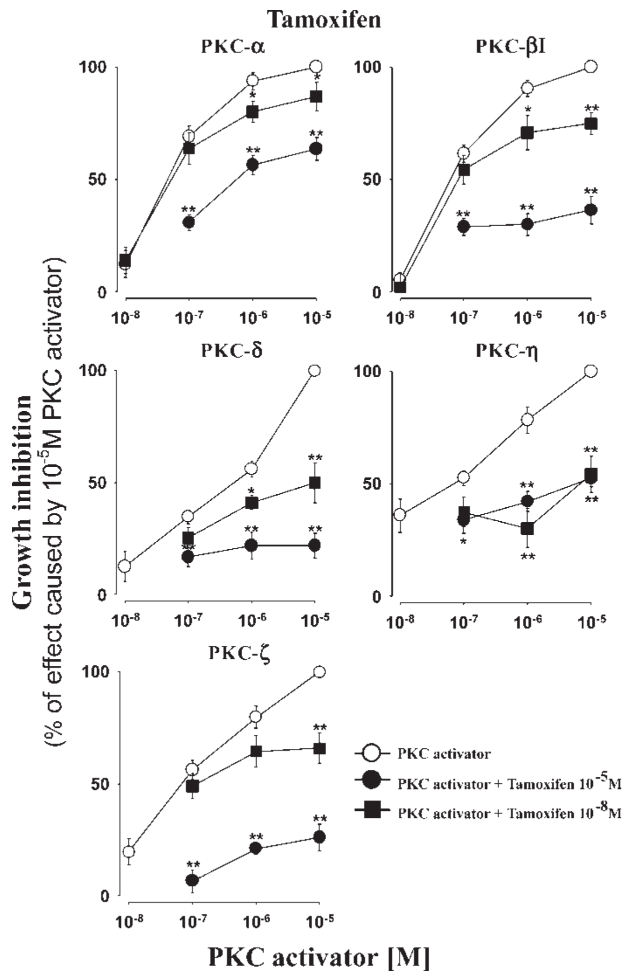

FIGURE 6 Interaction experiments for tamoxifen: concentration-response curves for PKC activator alone (open circles) and in the presence of 10^{-5}M (filled circles) or 10^{-8}M (filled squares) tamoxifen. Results are expressed as % of the maximal effect caused by 10^{-5}M PKC activator (PMA for PKC-α, -βI, -δ and -η; arachidonic acid for PKC-ζ). Shown are means ± SEM of 16–20 determinations. Significantly different from growth inhibition caused by PKC activator alone: *$P < 0.05$, **$P < 0.001$ (unpaired Student's *t* test).

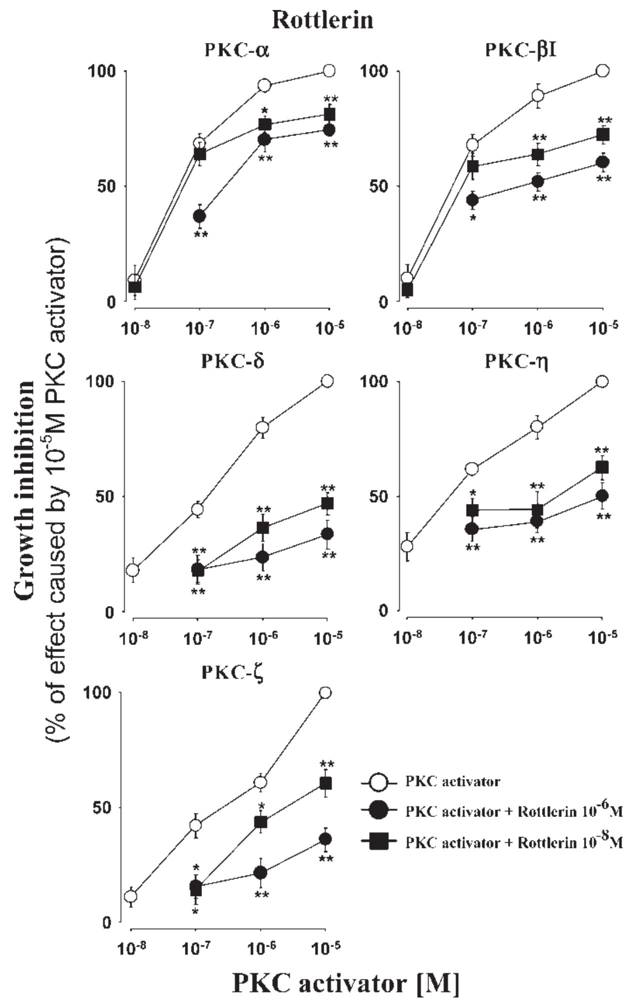

FIGURE 7 Interaction experiments for rottlerin: concentration-response curves for PKC activator alone (open circles) and in the presence of 10^{-6}M (filled circles) or 10^{-8}M (filled squares) rottlerin. Results are expressed as % of the maximal effect caused by 10^{-5}M PKC activator (PMA for PKC-α, -βI, -δ and -η; arachidonic acid for PKC-ζ). Shown are means ± SEM of 16–20 determinations. Significantly different from growth inhibition caused by PKC activator alone: *$P < 0.05$, **$P < 0.001$ (unpaired Student's *t* test).

a concentration-dependent growth inhibition,[25,26] which was also observed in the present study and which occurred only when the mammalian PKC isoform was expressed. PKC inhibitors reduced the growth inhibition,[24,27] which was also observed in the present study and for all isoforms expressed.

The attempt to compare the potency of each PKC inhibitor was based on the reduction of the effect of a PKC activator caused by a given concentration of PKC inhibitor. For each PKC isoform, estimation of PKC inhibitor potency was based on the shift to the right they caused of the concentration-response curve to the appropriate PKC activator (PMA for PKC-α, -βI, -δ and -η; arachidonic acid for PKC-ζ). The shift to the right was estimated at the EC_{50} level, and expressed as EC_{50} ratios (see Methods). The same concentration (10^{-8}M) was used for all PKC inhibitors. This concentration was chosen because at this concentration none of the PKC inhibitors altered, *per se*, growth of yeast expressing PKC-α, -βI, -δ, -η or -ζ. By using the same concentration of PKC inhibitor on all isoforms, it was possible not only to compare the potency of a given PKC inhibitor on the isoforms expressed, but also to compare the potency of the inhibitors tested on a given PKC isoform.

Among the PKC inhibitors studied, NPC 15437 was the compound that caused the highest EC_{50} ratio (observed in yeast expressing PKC-η). Therefore, this set of results may indicate that NPC 15437 is a selective PKC-η inhibitor. NPC 15437 has been described as a weak PKC inhibitor.[4] However, in our model NPC 15437 showed an extraordinary potency on PKC-η. A possible explanation for this discrepancy is the fact that PKC-η is present mainly in epithelial tissues,[1,31] which are not frequently used. Furthermore, mixtures of PKC isoforms, used for

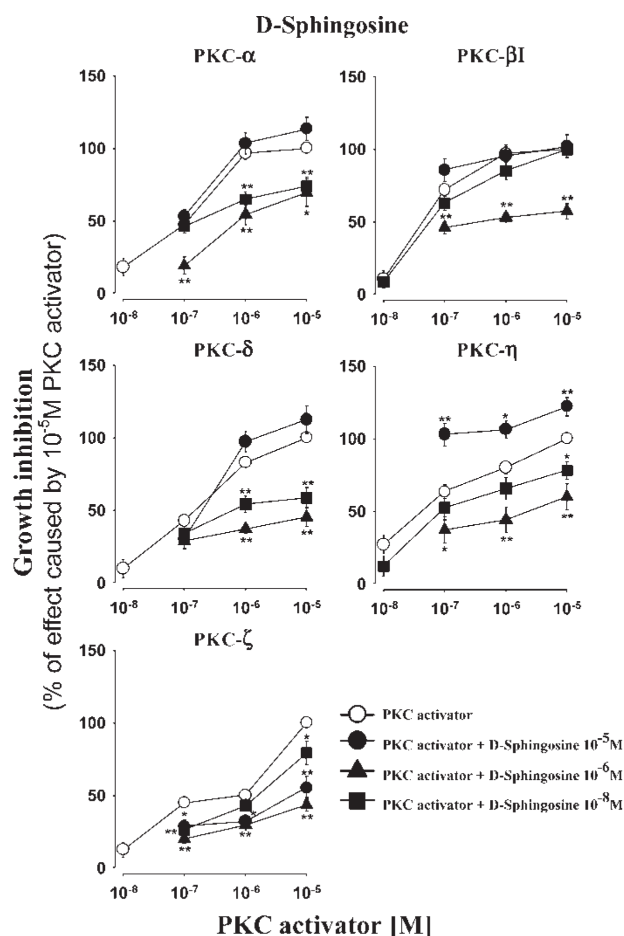

FIGURE 8 Interaction experiments for D-sphingosine: concentration-response curves for PKC activator alone (open circles) and in the presence of 10^{-5} M (filled circles), 10^{-6} M (filled triangles) or 10^{-8} M (filled squares) D-sphingosine. Results are expressed as % of the maximal effect caused by 10^{-5} M PKC activator (PMA for PKC-α, -βI, -δ and -η; arachidonic acid for PKC-ζ). Shown are means ± SEM of 16–20 determinations. Significantly different from growth inhibition caused by PKC activator alone: $*P < 0.05$, $**P < 0.001$ (unpaired Student's *t* test).

in vitro assays, are usually obtained from rat brain extracts, which contain small amounts of this isoform,[1] justifying the reported weak inhibition for NPC 15437.

Ro 32-0432 was the second compound that caused the highest EC_{50} ratios, again in yeast expressing PKC-η, but closer than those obtained on PKC-δ, -ζ and -βI. On PKC-α, Ro 32-0432 caused lower EC_{50} ratios, which differs from the results obtained in *in vitro* studies that suggested that Ro 32-0432 was more potent on PKC-α than on PKC-β.[14]

The third highest EC_{50} ratio was caused by rottlerin (observed in yeast expressing PKC-δ). This highest ratio observed on PKC-δ is in agreement with previous studies that showed that rottlerin was a potent inhibitor of PKC-δ.[21] However, the claimed selectivity for this isoform is challenged in the present study since the EC_{50} ratio obtained in yeast expressing PKC-η was similar to that obtained in yeast expressing PKC-δ.

Tamoxifen is an antitumor compound whose main mechanism of action is the blockade of estrogen receptors.[32,33] It has also been claimed that tamoxifen inhibits PKC.[16–20] The results obtained in the present study confirm that tamoxifen is a PKC inhibitor, since it reduced the effects of PKC activators on all PKC isoforms (see Figure 6). Although an estrogen-binding protein is also present in yeast, it is unlikely that reduction of the effect of PKC activator is due to an interference with this yeast protein, because tamoxifen did not cause any effect on non-transformed yeast, and furthermore it is known that tamoxifen has a negligible affinity for the estrogen-binding protein present in *S. cerevisiae*.[34] As observed with Ro 32-0432, tamoxifen caused the highest EC_{50} ratios on yeast expressing nPKC isoforms, and presented more marked differences than Ro 32-0432 between PKC-η and PKC-ζ or PKC-βI (EC_{50} ratios about 56 and 70 times lower than that of PKC-η, respectively).

As observed with rottlerin, tamoxifen did not caused significant EC_{50} ratios in yeast expressing PKC-α. These results must be interpreted with caution and should not be regarded as definitive proof that rottlerin and tamoxifen are inactive on PKC-α. The present comparison was based on results obtained with a single (and low) concentration of

TABLE II EC_{50} ratios for PKC inhibitors on the individual PKC isoforms tested

	EC_{50} Ratio[a]				
Compound	PKC-α	PKC-βI	PKC-δ	PKC-η	PKC-ζ
Chelerythrine	1.9 ± 0.3#	2.9 ± 0.3#	1.3 ± 0.1#	63.2 ± 1.7	1.4 ± 0.2#
NPC 15437	5.1 ± 0.5#	3.7 ± 0.3#	2.4 ± 0.1#	556.0 ± 44.7	4.5 ± 0.1#
Ro 32-0432	1.3 ± 0.2#	20.0 ± 3.1#	70.9 ± 7.4#	125.9 ± 11.1	25.2 ± 2.8#
Tamoxifen	ND	1.6 ± 0.2#	20.0 ± 2.7#	112.1 ± 9.7	2.0 ± 0.2#
Rottlerin	ND	1.4 ± 0.2#	70.9 ± 9.6	50.3 ± 6.8	8.9 ± 1.5#
D-Sphingosine	1.6 ± 0.2	1.3 ± 0.2	6.3 ± 1.2#	2.0 ± 0.3	1.6 ± 0.3

[a] EC_{50} ratio = EC_{50} (PKC activator + 10^{-8} M PKC inhibitor) / EC_{50} (PKC activator). The EC_{50} values were 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 mean ± SEM of 16–20 determinations. *ND*: Not determined (identical EC_{50} values in the absence or in the presence of the compound). Significant differences: from PKC-η, $^{\#}P < 0.05$ (one way ANOVA, followed by Tukey's *post-hoc* test).

these compounds. However, inhibition of PKC-α occurred with higher concentrations.

Although chelerythrine has been the most widely used PKC inhibitor, the isoform-selectivity of this compound was studied, for the first time, in the present work and the much higher EC_{50} ratios caused on yeast expressing PKC-η may indicate some selectivity for this isoform.

D-sphingosine was the compound that caused more similar EC_{50} ratios on all PKC isoforms tested, which may be taken as an indication that it is the least isoform-selective PKC inhibitor among the compounds tested. D-sphingosine has been considered as a potent PKC inhibitor.[23] However, the EC_{50} ratios obtained with this compound were among the lowest obtained, which questions the claimed high potency of D-sphingosine as a PKC inhibitor.

Inhibition of PKC activity by interfering with the regulatory domain was claimed to be an approach to obtain more selective PKC inhibitors.[12] Therefore, the question arises whether the site of action (regulatory or catalytic domain) is important for isoform-selectivity. The obtained results hardly support the assumption that PKC inhibitors acting at the regulatory domain may be more isoform-selective. In fact, chelerythrine, which acts at the catalytic domain, seems to have a more marked selectivity than D-sphingosine, which acts at the regulatory domain.

Considering the co-existence of several PKC isoforms in the same cell, an important aim would be to obtain and identify isoform-selective PKC inhibitors. The present results suggest that inhibition of PKC-η would be possible without a significant inhibition of other PKC isoforms (by using NPC 15437, for instance). It is further suggested that it would be possible to inhibit nPKC isoforms, without a significant inhibition of cPKC and aPKC isoforms (by using rottlerin, for instance). However, it is clear that more isoform-selective PKC inhibitors are required in order to selectively inhibit PKC isoforms other than PKC-η and to better clarify the physiological and pathophysiological roles of each PKC isoform. An analysis of the structural differences between different groups of PKC inhibitors acting at the regulatory domain and at the catalytic domain may provide important information on the molecular changes needed to obtain isoform-selective PKC inhibitors.

In conclusion, the present study is an attempt to describe the isoform-selectivity of six widely used PKC inhibitors by using an assay in which a single mammalian PKC isoform was expressed. It is revealed that PKC-η is the isoform that may be more selectively inhibited (namely by using NPC 15437) and that the PKC domain involved in the inhibition does not seem to be relevant to obtaining more isoform-selective PKC inhibitors.

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