Selective activation of protein kinase C-δ and -ε by 6,11,12,14-tetrahydroxy-abieta-5,8,11,13-tetraene-7-one (coleon U)

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Abstract

6,11,12,14-tetrahydroxy-abieta-5,8,11,13-tetraene-7-one (coleon U) is a diterpene compound isolated from *Plectranthus grandidentatus* with an antiproliferative effect on several human cancer cell lines. Herein, we studied the modulatory activity of coleon U on individual isoforms of the three protein kinase C (PKC) subfamilies, classical (cPKC-α and -βI), novel (nPKC-δ and -ε) and atypical (aPKC-ζ), using a yeast PKC assay. The results showed that, whereas the PKC activator phorbol-12-myristate-13-acetate (PMA) activated every PKC tested except aPKC, coleon U had no effect on aPKC and cPKCs. Besides, the effect of coleon U on nPKCs was higher than that of PMA. This revealed that coleon U was a potent and selective activator of nPKCs. The isoform-selectivity of coleon U for nPKC-δ and -ε was confirmed using an *in vitro* PKC assay. Most importantly, while PMA activated nPKCs inducing an isoform translocation from the cytosol to the plasma membrane and a G2/M cell cycle arrest, coleon U induced nPKCs translocation to the nucleus and a metacaspase- and mitochondrial-dependent apoptosis. This work therefore reconstitutes in yeast distinct subcellular translocations of a PKC isoform and the subsequent distinct cellular responses reported for mammalian cells. Together, our study identifies a new isoform-selective PKC activator with promising pharmacological applications. Indeed, since coleon U has no effect on cPKCs and aPKC, recognised as anti-apoptotic proteins, and selectively induces an apoptotic pathway dependent on nPKC-δ and -ε activation, it represents a promising compound for evaluation as an anti-cancer drug.

1. Introduction

6,11,12,14-tetrahydroxy-abieta-5,8,11,13-tetraene-7-one (coleon U) is an abietane diterpene compound isolated from *Plectranthus grandidentatus*, shown to inhibit the growth of several human cancer cell lines such as MCF-7 (breast), NCI-H460 (lung), SF-268 (CNS), TK-10 (renal) and UACC-62 (melanoma) in a dose-dependent manner [1]. However, the mechanisms of action responsible for this coleon U-induced growth inhibition has not been elucidated. In a later study, a dose-dependent antiproliferative effect of coleon U on T- and B-lymphocyte cells was also reported [2]. Although this study established a relationship between the antiproliferative effect of coleon U and its capacity to induce apoptosis in lymphocyte cells, the molecular mechanisms associated with its antiproliferative effect remained unclear. However, the interference of this small-molecule with some cell signalling transduction

kinases, such as protein kinase C (PKC) and/or protein tyrosine kinases, was hypothesised as a possible mechanism responsible for coleon U-induced growth inhibition [2]. Since several diterpene compounds, such as phorbol esters, represent potent PKC activators [3,4], we questioned whether coleon U could indeed modulate PKC activity.

PKC is considered an important family of signalling serine/threonine kinases with at least 10 isoforms grouped into three subfamilies based on their primary structure and cofactors required for activation: the classical PKCs (cPKCs: α, βI, βII and γ), activated by the second messengers Ca^{2+} and diacylglycerol (DAG), the novel PKCs (nPKCs: δ, ε, η and θ) which respond only to DAG and the atypical PKCs (aPKCs: ζ and λ\ι) not responsive to either of the second messengers [4]. The PKC family is responsible for regulating a variety of physiological processes such as differentiation, proliferation, cell cycle and apoptosis, in an isoform specific manner. Hence, PKC isoforms represent key pharmacological targets for the treatment of numerous pathologies. For instance, some PKC isoforms have been recognised as important players in carcinogenesis, rendering them potentially suitable targets for anti-cancer therapy [3,5–7]. While PKC-α, -β and -ζ are frequently associated with proliferative effects being involved in the tumorigenesis of various cancers, PKC-δ is often linked to anti-proliferative/pro-apoptotic effects in mammals [3,5–7]. Consequently, selective activators of PKC-α, -β and -ζ are considered tumour promoters, while selective activators of PKC-δ represent promising anti-cancer drugs [3,5–7]. Although PKC-δ and -ε display a high degree of homology and similar substrate specificity, suggesting similar targets in signal transduction pathways for both nPKCs, they are frequently described as mediating quite contrasting physiological effects [3,5,6]. However, although several studies suggest that PKC-ε favours life over death [3,5–7], recent research works also showed that PKC-ε activation can contribute to apoptosis. For example, it was demonstrated that ethanol induces apoptosis in hepatocytes via activation of the nPKC isoforms, PKC-δ and -ε[8].

In order to achieve the regulation of a particular PKC isoform, without affecting the activity of other isoforms also present in the cell, PKC isoform-selective activators are required. However, the complexity of the PKC family and the difficulty in carrying out independent analysis of an individual PKC isoform in mammalian cells may justify the low number of PKC isoform-selective modulators identified until now [3,7,9].

Based on these data, we exploited a yeast PKC expression system to study the modulatory activity of coleon U on individual PKC isoforms of the three PKC subfamilies, cPKCs (α and βI), nPKCs (δ and ε) and aPKCs (ζ), considered as major isoforms in carcinogenesis. The conservation of many pathways and cellular processes in yeast, such as apoptosis [10], has allowed transposing the knowledge obtained in yeast to mammalian cells and vice-versa. Additionally, yeast expressing human proteins has been used as a valuable tool to elucidate the role of these proteins in complex cellular processes, and to screen for their pharmacological modulators [11,12]. Furthermore, since the yeast PKC (Pkc1p in *Saccharomyces cerevisiae*) is a structural but not a functional homologue of mammalian PKC isoforms [13], yeast has been considered a well-suited organism to study individual mammalian PKC isoforms. In fact, it was demonstrated that mammalian PKCs expressed in yeast have functional characteristics similar to those found in mammalian cells [14,15]. It was also shown that the yeast PKC expression system was a potential *in vivo* assay for the screening of PKC modulators [14,16]. Based on these data, this yeast PKC assay has been used by our group not only to study the role of PKC isoforms on apoptosis regulation [17], but also to search for isoform-selective PKC activators [18,19] and inhibitors [20,21]. This simpler eukaryotic cell model, established and validated in such studies, allows the effect of small-molecules on each PKC isoform to be analysed

without the genetic complexity of the PKC family, specifically the coexistence of multiple PKC isoforms in the same mammalian cells and the extensive cross-talk amongst numerous mammalian signalling pathways.

In the present study, we found that coleon U is a potent and selective activator of nPKC-δ and -ε. Besides, several experiments were carried out in order to elucidate the molecular mechanisms of action behind the observed coleon U-induced growth inhibition in yeast expressing the nPKC-δ or -ε. Another relevant point arising from this work was the validation of the yeast assay to reconstitute the distinct subcellular translocations of a specific mammalian PKC isoform and the subsequent different cellular responses described in higher eukaryotes.

2. Materials and methods

2.1. Plant material, isolation and identification of coleon U

Details concerning the extraction, isolation and structure elucidation of the abietane diterpene coleon U from *P. grandidentatus* Gürke (Fig. 1) have previously been described [1,22].
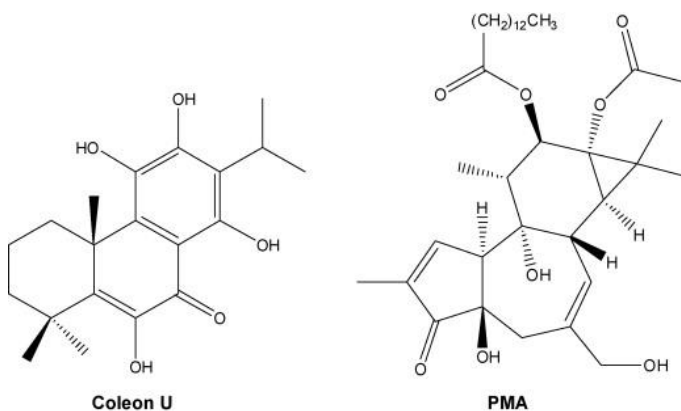

Fig. 1. Chemical structures of coleon U, an abietane diterpene isolated from *P. grandidentatus*, and phorbol 12-myristate 13-acetate (PMA).

2.2. Plasmids

Constructed yeast expression plasmids YEplac181-*LEU2* with the cDNA encoding for bovine PKC-α, rat PKC-βI or PKC-δ, mouse PKC-ε or PKC-ζ, under control of a galactose-inducible *GAL1* promoter, were kindly provided by Dr. Nigel Goode (The Royal Veterinary College, Hawkshead Lane, Hertfordshire, UK). Constructed yeast expression plasmid pOW4-*URA3* with the cDNA encoding for human Bcl-xL, under control of an *ADH1* promoter, was kindly provided by Dr. Charles Rudin (Cancer Research Building, Baltimore, USA). Constructed yeast expression plasmid pCLbGFP-*TRP3* with the cDNA encoding for mitochondria-localised green fluorescent protein (mt-GFP), under control of a *GAL1-10* promoter, was kindly provided by Dr. Stéphen Manon (Université de Bordeaux, Bordeaux, France). All the plasmids used were amplified in *Escherichia coli* DH5α and confirmed by restriction analysis.

2.3. Yeast strain, growth conditions and yeast expression of a mammalian protein

For yeast expression studies *Saccharomyces cerevisiae* strain CG379 (*α ade5 his7-2 leu2-112 trp1-289α ura3-52* [*Kil-O*]; Yeast Genetic Stock Center, University of California, Berkeley, USA) was transformed as reported [17]. To ensure selection of transformed yeast, cells were routinely grown in a minimal selective medium. To induce yeast expression of a mammalian protein, cells were diluted to 0.05 measured at 600 nm (OD_{600}; Jenway 6310 Spectrophotometer, Jenway, Felsted, Dunmow, Essex, UK) in a 2% (w/v) galactose and raffinose (Sigma–Aldrich, Sintra, Portugal) selective medium and grown, at 30 °C under continuous shaking, to 0.5 OD_{600} (mid-log phase; about 42 h incubation), as described [17]. Yeast expression of a mammalian PKC isoform and/or human Bcl-xL was previously confirmed by Western blot analysis [17,19].

2.4. Effect of compounds on yeast cell growth

All compounds tested were prepared in dimethyl sulfoxide (DMSO; Sigma–Aldrich, Sintra, Portugal). To analyse the effect of compounds on yeast growth, transformed cells were incubated in galactose selective medium with 0.1–10 µM coleon U, 1 µM phorbol 12-myristate 13-acetate (PMA; Sigma–Aldrich, Sintra, Portugal) or 0.1% DMSO only to 0.5 OD_{600}. Cell growth was determined by colony forming unit (c.f.u.) counts, as described [17]. The percentage of growth inhibition was estimated considering 100% growth as the number of c.f.u. obtained with yeasts incubated with DMSO only.

2.5. *In vitro* PKC assay

The *in vitro* PKC assay was performed using the non-radioactive kit MESACUP Protein Kinase Assay System and purified PKC enzymes from Upstate (Grupotaper, Sintra, Portugal): cPKCs (mixture of classical PKC isoforms, α, β and γ, obtained from rat brain; 5 ng/assay); nPKC-δ (50 ng/assay); nPKC-ε (12.5 ng/assay); aPKC-ζ (12.5 ng/assay), basically according to the kit procedure. Briefly, this ELISA-based detection method uses a peptide pseudosubstrate, pre-coated on a 96-well plate that can be phosphorylated by PKC. Samples containing the active PKC enzyme were transferred to the pseudosubstrate-coated wells. A biotinylated monoclonal antibody that recognises the phosphorylated form of the pseudosubstrate was added to the wells and detected using HRP-conjugated streptavidin. Colour intensity of reaction mixtures containing the endogenous PKC activator phosphatidylserine (PS), with or without 0.3 µM coleon U, was determined photometrically at 490 nm using a microplate spectrofluorometer (PowerWave™ S Microplate Spectrophotometer, Bio-TEK instruments, Inc., Highland Park, Winooski VT, USA). PKC activity is directly proportional to colour intensity. OD_{490} obtained with the reaction mixture containing PS only (control) was considered as 100% PKC activation.

2.6. Cell cycle analysis

Flow cytometric analysis of DNA content was obtained using Sytox Green Nucleic Acid from Molecular Probes (Alfagene, Carcavelos, Portugal), basically as reported [23]. Briefly, about 10^7 cells incubated in galactose selective medium with 1 µM PMA, 1 µM coleon U or DMSO only were fixed in 70% (v/v) ethanol overnight at 4 °C, treated with 250 µg/ml RNase A (DNase-free; Sigma–Aldrich, Sintra, Portugal) for 3 h at 50 °C and thereafter with 1 mg/ml Proteinase K (Sigma–Aldrich, Sintra, Portugal) for 3 h at 37 °C. Subsequently, cells were incubated with 10 µM Sytox Green overnight at 4 °C. Fluorescence from at least 30,000 cells was analysed using the FL1 detector in linear

amplification from a flow cytometer. Yeast cell cycle phases were quantified using ModFit LT™ software (Verity Software House, Inc., Topsham, USA).

2.7. Analysis of plasma membrane integrity, DNA fragmentation and chromatin condensation

Propidium iodide (PI) and TUNEL staining to monitor plasma membrane integrity and DNA fragmentation respectively were carried out as described [17]. Briefly, about 10^7 cells incubated in galactose selective medium with 1 µM coleon U or DMSO only were collected and incubated with 5 µg/ml PI (Sigma–Aldrich, Sintra, Portugal) for 10 min at room temperature. TUNEL was performed using the In Situ Cell Death Detection Kit, Fluorescein (Roche Diagnostics, Amadora, Portugal). For chromatin condensation analysis, cells were incubated with 4 µg/ml 4,6-diamido-2-phenyl-indole (DAPI; Sigma–Aldrich, Sintra, Portugal) for 15 min at room temperature, as described [24]. At least 600 cells per sample were analysed under a fluorescence microscope.

2.8. Assessment of yeast metacaspase activity

Yeast metacaspase (Yca1p) activation was analysed basically as described [17]. Flow cytometric analysis: about 10^6 cells incubated in galactose selective medium with 1 µM coleon U or DMSO only were collected and incubated with 12.5 µM of FITC-VAD-fmk from Promega (VWR International Material de Laboratório, Lda., Lisboa, Portugal) for 1 h at 30 °C; fluorescence from at least 10,000 cells was analysed using the FL1-H detector from a flow cytometer. Cell death assay: cells were incubated in galactose selective medium with 1 µM coleon U and 20 µM of the caspase inhibitor z-VAD-fmk from Promega (VWR International Material de Laboratório, Lda., Lisboa, Portugal). Cell death was assessed by c.f.u. counts, considering 100% survival as the number of c.f.u. obtained with cells incubated with DMSO only.

2.9. Assessment of reactive oxygen species (ROS) production

ROS production was monitored by flow cytometry using dihydroethidium (DHE) from Molecular Probes (Alfagene, Carcavelos, Portugal) as described [25]. About 10^6 cells, incubated in galactose selective medium with 1 µM coleon U or DMSO only, were collected and incubated with 5 µg/ml DHE for 30 min at 30 °C. Fluorescence from at least 10,000 cells was analysed using the FL2-H detector from a flow cytometer.

2.10. Assessment of mitochondrial membrane potential ($\Delta\psi_m$)

Modification of $\Delta\psi_m$ in transformed yeast was monitored by flow cytometry using 3,3'-dihexyloxacarbocyanine iodide (DiOC$_6$(3)) from Molecular Probes (Alfagene, Carcavelos, Portugal). About 10^6 cells, incubated in galactose selective medium with 1 µM coleon U or DMSO only were incubated with 1 nM DiOC$_6$(3) for 30 min at 30 °C. Thereafter, 2 µg/ml PI was added to exclude necrotic cells. Carbonyl cyanide 3-chlorophenylhydrazone (CCCP; Sigma–Aldrich, Sintra, Portugal) was used as positive control; after treatment with DiOC$_6$(3), cells incubated with DMSO only were treated with 0.4 nM CCCP for 15 min at 30 °C. Fluorescence from at least 10,000 cells was analysed using the FL1-H and FL2-H detectors from a flow cytometer.

2.11. Assessment of mitochondrial fragmentation

Analysis of mitochondrial fragmentation of transformed yeast was carried out using the plasmid pCLbGFP encoding for mt-GFP, as described [26]. Cells incubated in galactose selective medium with 1 µM coleon U or DMSO only were observed under a fluorescence microscope.

2.12. Immunofluorescence assay

About 10^7 cells grown in galactose selective medium with 1 µM PMA, 1 µM coleon U or DMSO only were fixed with 3.7% formaldehyde (Sigma–Aldrich, Sintra, Portugal) for 30 min at 30 °C. For spheroplasts formation, cells were treated with zymolyase solution, containing 0.1 M potassium phosphate pH 7.5, 2 µl/ml 2-mercaptoethanol (Sigma–Aldrich, Sintra, Portugal), 1 mg/ml zymolyase 20 T (MP Biomedicals Solon, Fountain Parkway Solon, USA), at 37 °C for 25 min. Spheroplasts were transferred to polylysine-coated glass slides (Sigma–Aldrich, Sintra, Portugal) and permeabilised with 1% Triton X-100 (Sigma–Aldrich, Sintra, Portugal) for 2 min at 4 °C before incubation with the mouse monoclonal antibody anti-PKC-δ or anti-PKC-ε (1:50) from Santa Cruz Biotechnology (Frilabo, Porto, Portugal) for 2 h at room temperature. This was then followed by incubation with the anti-mouse Alexa Fluor 488 secondary antibody (1:200) from Molecular Probes (Alfagene, Carcavelos, Portugal) for 2 h at room temperature. Mounting medium containing 1.5 µg/ml DAPI was used to visualise nuclear DNA. Samples were observed under a fluorescence microscope.

2.13. Flow cytometric data acquisition and analysis

Flow cytometric analysis was performed using a FACSCalibur™ flow cytometer and the CellQuest software (BD Biosciences, San José, CA, USA).

2.14. Fluorescence microscopy

For fluorescent microscopic examination, samples were observed under an Eclipse E400 fluorescence microscope (Nikon, Japan) equipped with a 100 W mercury lamp and appropriate filter setting. Yeast cells were observed with an oil immersion lens (Plan Fluor 100/1.30) and images were captured by a Digital Sight camera system (Nikon DS-5Mc, Japan) carrying built-in software for image acquisition (Nikon ACT-2U, Japan).

2.15. Statistical analysis

Data were analysed statistically using the SigmaStat 3.1 programme (SYSTAT®Software, Inc., Mountain River, CA, USA). Differences between means were tested for significance using the unpaired Student's *t*-test. *P* values of 0.05 or lower were considered statistically significant. Results are expressed as the mean ± S.E.M. of the indicated number of experiments.

3. Results

3.1. Coleon U is a potent and selective activator of nPKC-δ and -ε

In order to analyse a possible modulation of PKC isoforms by coleon U (Fig. 1), we used a yeast PKC expression system that consists of the use of yeast cells expressing an individual mammalian PKC-α, -βI, -δ, -ε or -ζ. In this assay, it was demonstrated that PKC activators inhibit the growth of yeast expressing a PKC isoform without interfering with the growth of yeast transformed with the empty vector (control yeast) [14,18,19].

Hence, we began by analysing the effect of several concentrations of coleon U, 0.1–10 µM, on the growth of yeast expressing PKC-α, -βI, -δ, -ε or -ζ and control yeast (Fig. 2A). Yeast cell growth was analysed by c.f.u. counts and

the effects obtained with coleon U were compared to those obtained with the standard PKC activator, PMA (Fig. 1; an activator of cPKCs and nPKCs). We detected that 0.1 and 1 µM coleon U significantly inhibited the growth of yeast expressing PKC-δ or -ε without affecting the growth of control yeast and yeast expressing PKC-α, -βI or -ζ (Fig. 2A). This selectivity of coleon U to inhibit the growth of yeast expressing PKC-δ or -ε was particularly evident for 1 µM (Fig. 2B). In fact, for this concentration, and in opposition to PMA that activated every PKC tested except aPKC-ζ, coleon U had no effect on cPKCs as well as on aPKC-ζ (Fig. 2B and C). In addition, the effect of coleon U on yeast expressing the nPKCs, PKC-δ or -ε, was shown to be higher than that obtained with PMA (Fig. 2B and C). These results therefore indicated that coleon U was a potent and selective activator of the two nPKCs, PKC-δ and -ε.
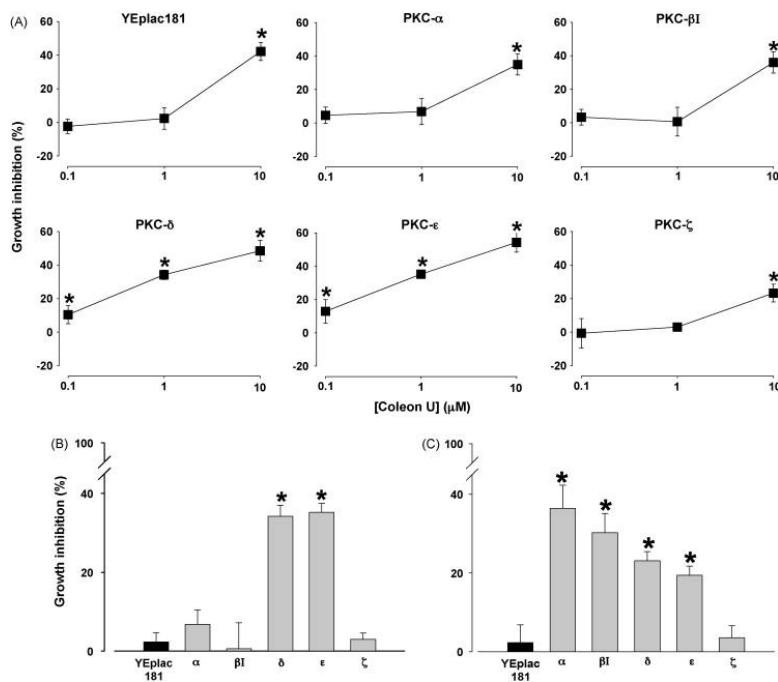

Fig. 2. Effect of PMA and coleon U on the growth of control yeast and yeast expressing PKC-α, -βI, -δ, -ε or -ζ. Transformed yeast cells were diluted to 0.05 OD_{600} in galactose selective medium and grown with coleon U, PMA or DMSO only to 0.5 OD_{600} (about 42 h incubation). The percentage of growth inhibition was estimated by c.f.u. counts, considering 100% growth as the number of c.f.u. obtained with yeast incubated with DMSO only. (A) Concentration–response curves for the coleon U effect on the growth of transformed yeast. Effect of (B) 1 µM coleon U and (C) 1 µM PMA on the growth of transformed yeast. Data are the mean ± S.E.M. of four to eight independent experiments with six replicates each. Values significantly different from those obtained with (A) DMSO and (B, C) control yeast, $*P < = 0.001$.

Direct activation of PKC-δ and -ε by coleon U and the selectivity of this small-molecule for these nPKCs was further confirmed using an *in vitro* protein kinase assay system and purified PKC enzymes: cPKCs (mixture of classical PKC isoforms, α, β and γ), nPKC-δ, nPKC-ε and aPKC-ζ. In the *in vitro* PKC assay, PKC activators increase the phosphatidylserine (PS; an endogenous PKC activator) effect. In fact, in this assay, it was observed that 0.3 µM coleon U significantly increased the PS effect on nPKC-δ and -ε. In opposition, 0.3 µM coleon U did not interfere with the PS effect on cPKCs and aPKC-ζ (Fig. 3).
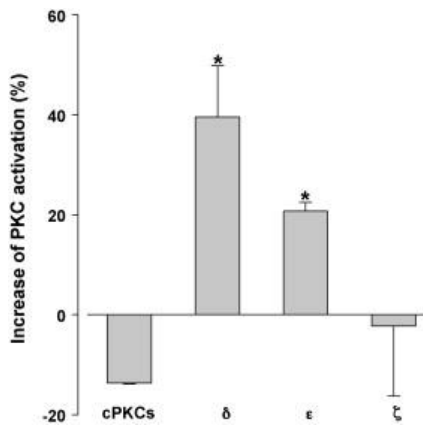
Fig. 3. *In vitro* PKC assay. Direct activation of nPKC-δ and -ε by coleon U and its selectivity for these nPKCs was confirmed *in vitro* using a protein kinase assay system and purified PKC enzymes: cPKCs (mixture of classical PKC isoforms, α, β and γ); nPKC-δ; nPKC-ε and aPKC-ζ. Coleon U was tested at the final concentration of 0.3 μM. Colour intensity of reaction mixtures containing the endogenous PKC activator phosphatidylserine (PS) with and without coleon U was determined photometrically at 490 nm using a microplate spectrofluorometer. Data represent the increase in the percentage of PKC activation obtained when coleon U was added to PS, considering 100% PKC activation the OD_{490} obtained with the reaction mixture containing PS only (control), and correspond to the mean ± S.E.M. of two independent experiments. Values significantly higher than those obtained with the control, *$P < 0.05$.

3.2. Coleon U-induced growth inhibition in yeast expressing nPKC-δ or -ε is associated with the occurrence of a metacaspase- and mitochondrial-dependent apoptotic cell death

It was shown that the expression of bovine PKC-α in *S. cerevisiae* causes growth inhibition associated with the occurrence of a G2/M arrest, which is markedly increased by treatment of these cells with PMA [15]. However, as previously reported by us [17], under our experimental conditions and with the yeast strain used, single expression of a mammalian PKC-α, -δ, -ε or -ζ in yeast did not significantly interfere with cell growth and survival. Even so, in agreement with that reported for PKC-α[15], treatment of yeast expressing nPKC-δ or -ε with PMA caused significant growth inhibition related to a G2/M cell cycle arrest (Fig. 4A and B). This blockage in cell cycle progression by PMA was not accompanied by the occurrence of cell death, as revealed by the preservation of plasma membrane integrity, the absence of DNA fragmentation and chromatin condensation in PMA-treated yeast cells (data not shown).
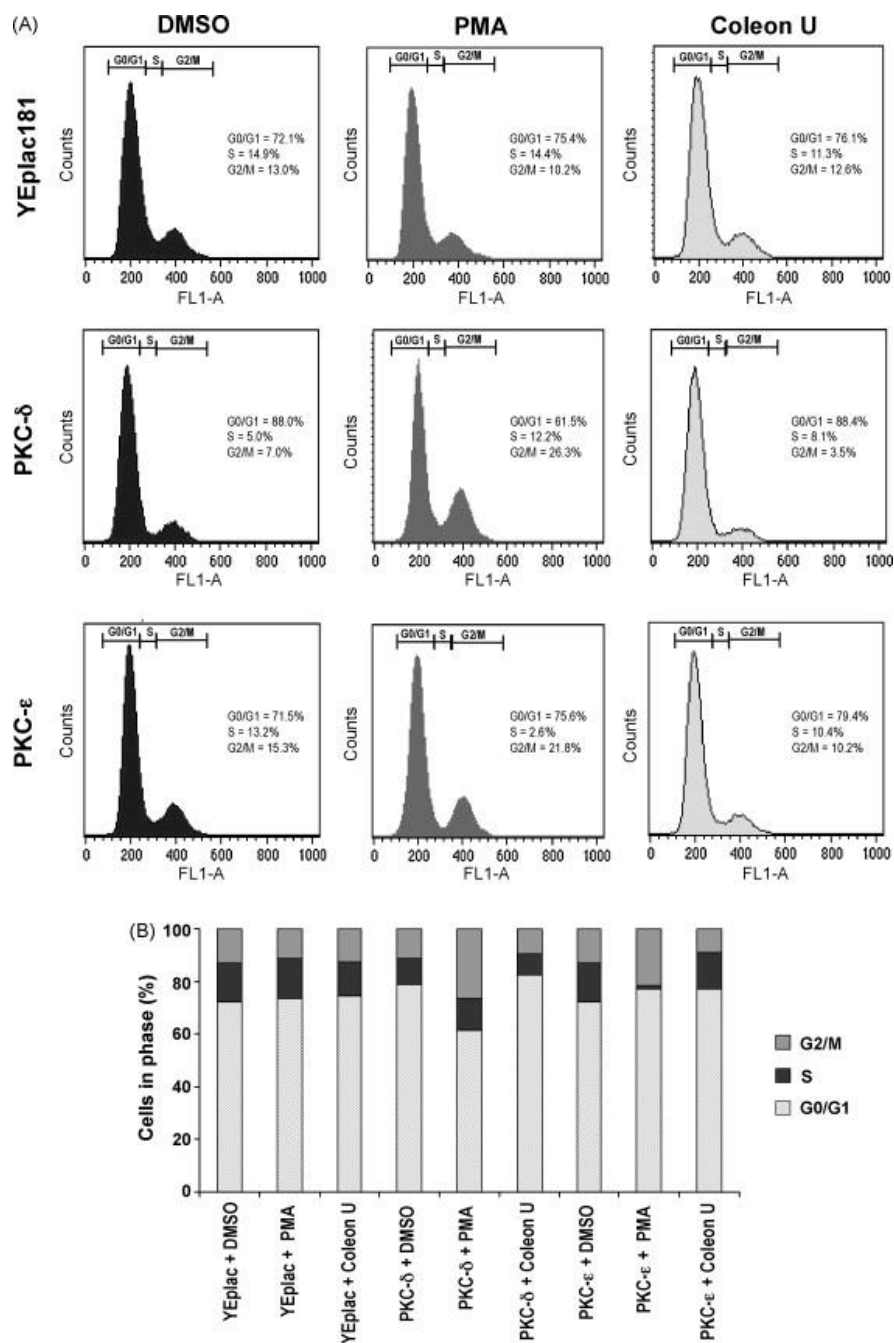
Fig. 4. Effect of PMA and coleon U on the cell cycle progression of control yeast and yeast expressing nPKC-δ or -ε. Transformed yeast cells were diluted to 0.05 OD_{600} in galactose selective medium and grown with 1 μM coleon U, 1 μM PMA or DMSO only to 0.5 OD_{600} (about 42 h incubation). Analysis of DNA content was carried out by flow cytometry using Sytox Green. (A) Histograms represent one of three independent experiments. (B) Quantification of yeast cell cycle phases; data are the mean of three independent experiments.

With a view to elucidating the molecular mechanisms behind coleon U-induced growth inhibition in yeast cells expressing nPKC-δ or -ε, we began by analysing the effect of this small-molecule on the cell cycle progression of these transformed yeast. In contrast to PMA, coleon U only slightly interfered with the cell cycle progression of yeast

expressing nPKC-δ or -ε (Fig. 4A and B). This small effect of coleon U on cell cycle could not explain *per se* the marked growth inhibition induced by this small-molecule on yeast expressing nPKC-δ or -ε.

Hence, we next addressed whether coleon U-induced growth inhibition could reflect cell death. With this goal, several apoptotic markers were investigated. We verified that 1 μM coleon U caused in yeast expressing nPKC-δ or -ε, but not in control yeast and yeast expressing PKC-α, -βI or -ζ, a marked increase in the percentage of cells with chromatin condensation and DNA fragmentation (TUNEL-positive cells), without loss of plasma membrane integrity as revealed by the low percentage of PI-positive cells (absence of necrosis) (Fig. 5A–C). These results indicated that coleon U-induced growth inhibition in yeast expressing PKC-δ or -ε was linked to the activation of an apoptotic cell death.
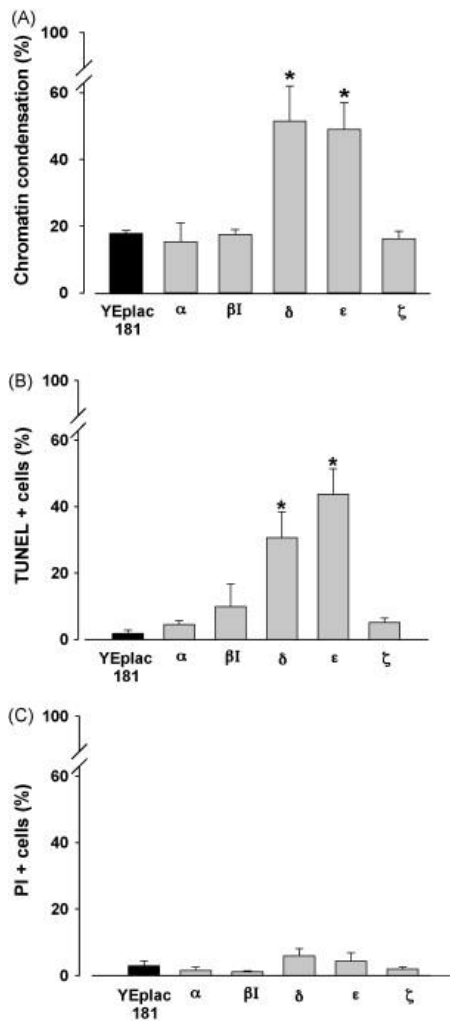

Fig. 5. Effect of coleon U on chromatin condensation, DNA fragmentation and plasma membrane integrity of control yeast and yeast expressing PKC-α, -βI, -δ, -ε or -ζ. Transformed yeast cells were diluted to 0.05 OD_{600} in galactose selective medium and grown with 1 μM coleon U to 0.5 OD_{600} (about 42 h incubation). (A) Chromatin condensation was analysed with DAPI. (B) Cells with DNA fragmentation are indicated as TUNEL + cells. (C) Necrotic cells are indicated as PI + cells. Data are the mean ± S.E.M. of three to four independent experiments; means correspond to

counts of at least 600 cells per sample analysed by fluorescence microscopy. Values significantly different from those obtained with the control yeast, *$P < 0.05$.

Additionally, flow cytometric analysis of FITC-VAD-fmk stained cells revealed the occurrence of yeast metacaspase (Yca1p) activation in coleon U-treated yeast cells expressing PKC-δ or -ε, but not in control yeast and yeast expressing PKC-α, -βI or -ζ (Fig. 6A and B). This was further confirmed by the marked decrease in the percentage of dead cells expressing nPKC-δ or -ε obtained when coleon U treatment was carried out in the presence of the caspase inhibitor z-VAD-fmk (Fig. 6C).
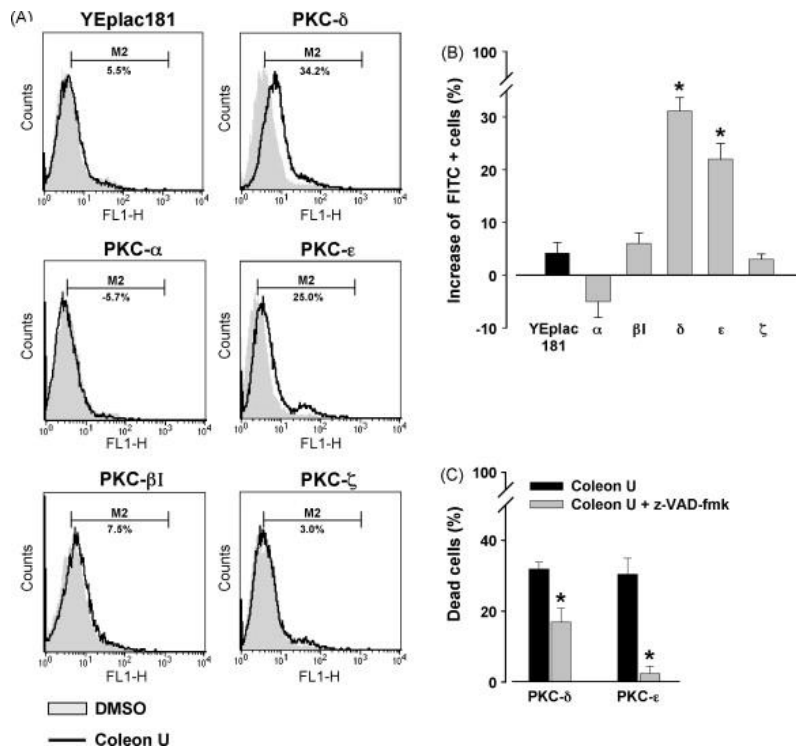

Fig. 6. Effect of coleon U on metacaspase activation of control yeast and yeast expressing PKC-α, -βI, -δ, -ε or -ζ. Transformed yeast cells were diluted to 0.05 OD$_{600}$ in galactose selective medium and grown with 1 μM coleon U or DMSO only to 0.5 OD$_{600}$ (about 42 h incubation). (A and B) Yeast metacaspase activation was monitored by flow cytometry using FITC-VAD-fmk. (A) Overlays of green fluorescence histograms were obtained with cells incubated with coleon U and DMSO only. M2 values correspond to the increase in the percentage of FITC-VAD-fmk positive cells obtained when cells were incubated with coleon U. Data represent one of two independent experiments. (B) Data are the mean ± S.E.M. of M2 values obtained from two independent experiments. Values significantly different from those obtained with the control yeast, *$P < 0.05$. (C) Effect of the caspase inhibitor z-VAD-fmk on the survival of coleon U-treated yeast cells expressing nPKC-δ or -ε. Cells were incubated in galactose selective medium with 1 μM coleon U, 1 μM coleon U and 20 μM of z-VAD-fmk or DMSO only to 0.5 OD$_{600}$. The percentage of dead cells was estimated by c.f.u. counts, considering 100% survival the number of c.f.u. obtained with cells incubated with DMSO only. Data are the mean ± S.E.M. of four independent experiments with six replicates each. Values significantly different from those obtained with yeast incubated with coleon U only, *$P < 0.05$.

We further observed that 1 µM coleon U markedly increased mitochondrial ROS production (Fig. 7A and B), decreased $\Delta\psi_m$ (Fig. 8A and B) and increased mitochondrial fragmentation (Fig. 8C) in yeast expressing nPKC-δ or -ε, but not in control yeast and yeast expressing PKC-α, -βI or -ζ.
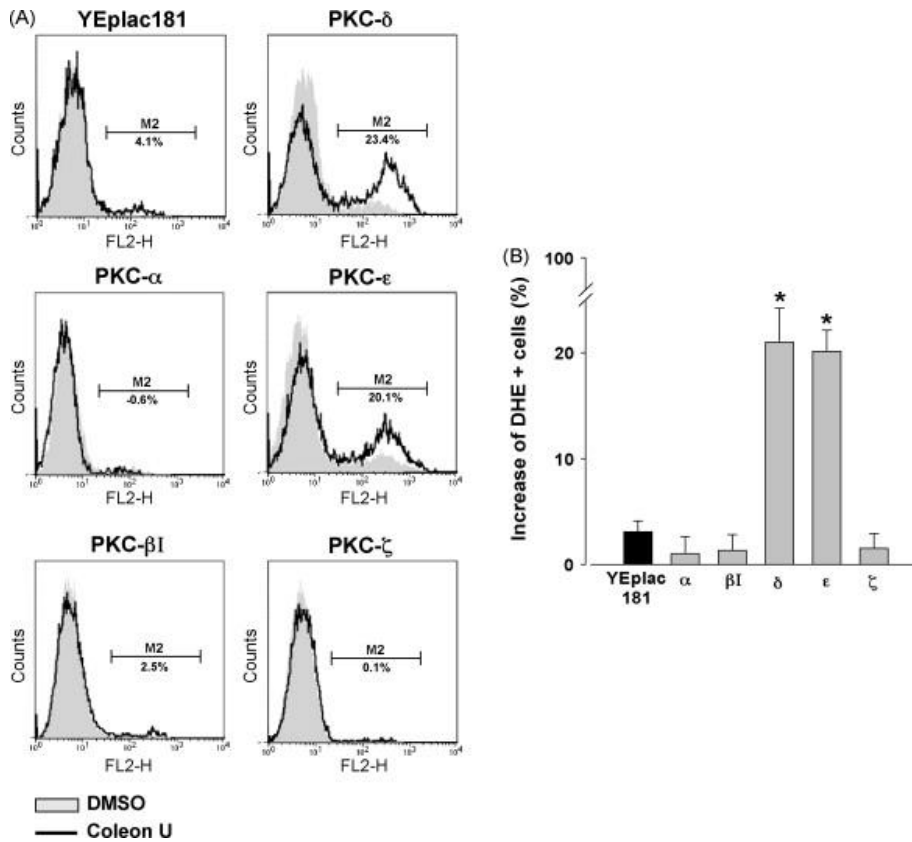
Fig. 7. Effect of coleon U on mitochondrial ROS production of control yeast and yeast expressing PKC-α, -βI, -δ, -ε or -ζ. Transformed yeast cells were diluted to 0.05 OD_{600} in galactose selective medium and grown with 1 µM coleon U or DMSO only to 0.5 OD_{600} (about 42 h incubation). ROS production was analysed by flow cytometry using DHE. (A) Overlays of red fluorescence histograms obtained with yeast incubated with coleon U or DMSO only. M2 values correspond to the increase in the percentage of DHE positive cells obtained when cells were incubated with coleon U. Data represent one of two independent experiments. (B) Data are the mean ± S.E.M. of M2 values obtained from two independent experiments. Values significantly different from those obtained with the control yeast, *$P < 0.05$.
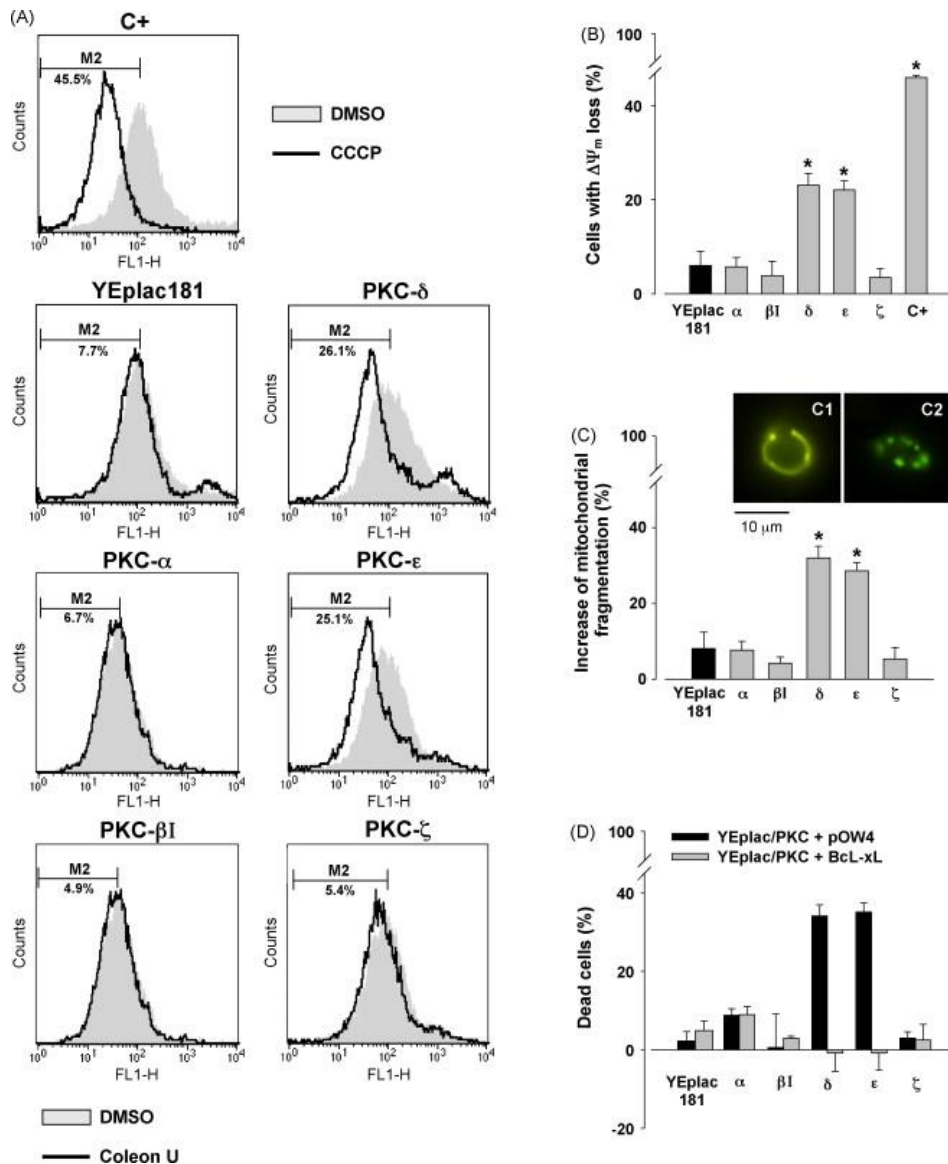
Fig. 8. Effect of coleon U on $\Delta\psi_m$ and mitochondrial fragmentation of control yeast and yeast expressing PKC-α, -βI, -δ, -ε or -ζ and interference of human Bcl-xL with the coleon U-induced yeast cell death. Transformed yeast cells were diluted to 0.05 OD_{600} in galactose selective medium and grown with 1 μM coleon U or DMSO only to 0.5 OD_{600} (about 42 h incubation). (A and B) $\Delta\psi_m$ was monitored by flow cytometry using $DiOC_6(3)$. (A) Overlays of green fluorescence histograms obtained with yeast incubated with coleon U and DMSO only. C + , positive control, control yeast treated with CCCP. M2 values correspond to the percentage of cells with $\Delta\psi_m$ loss obtained when cells were incubated with coleon U. Data represent one of two independent experiments. (B) Data are the mean ± S.E.M. of M2 values obtained from two independent experiments. Values significantly different from those obtained with the control yeast, *$P < 0.05$. (C) Mitochondrial fragmentation was analysed using yeasts expressing mt-GFP. Values correspond to the increase in the percentage of cells incubated with coleon U with mitochondrial fragmentation. Data are the mean ± S.E.M. of three independent experiments; means correspond to counts of at least 100 cells per sample analysed by fluorescence microscopy. Values significantly different from those obtained with the control yeast, *$P < 0.05$. C1: Normal tubular mitochondria; C2: Mitochondrial fragmentation observed with coleon U-treated

yeast expressing nPKC-δ or -ε. (D) The interference of Bcl-xL with the coleon U effect was analysed by c.f.u. counts, considering 100% survival as the number of c.f.u. obtained with DMSO. Data are the mean ± S.E.M. of four independent experiments with six replicates each.

It was showed that human Bcl-xL is also a mitochondrial anti-apoptotic protein in yeast, inhibiting yeast apoptosis induced by stress stimuli such as hydrogen peroxide, menadione [27] and acetic acid [17]. Moreover, a previous work performed by our group showed that co-expression of human Bcl-xL with a mammalian PKC isoform did not significantly interfere with yeast cell growth and survival [17]. Based on these data, the interference of human Bcl-xL with coleon U-induced cell death in yeast expressing PKC-α, -βI, -δ, -ε or -ζ and control yeast was analysed. The results obtained showed that Bcl-xL completely abolished the coleon U-induced apoptosis in yeast expressing nPKC-δ or -ε, without interfering with the coleon U effect in control yeast and yeast expressing PKC-α, -βI, -δ, -ε or -ζ (Fig. 8D).

Together, the results obtained corroborate the activation by coleon U of a metacaspase- and mitochondrial-dependent apoptotic pathway dependent on nPKC-δ or -ε expression in yeast.

3.3. Coleon U-induced apoptosis is associated with the translocation of nPKC-δ and -ε from the cytosol to the nucleus of yeast cells

In mammalian cells, it was reported that distinct subcellular translocations of a specific PKC isoform lead to different cellular responses [28]. Since PMA and coleon U induced in yeast expressing nPKC-δ or -ε different cellular responses, we questioned whether these two PKC activators could also induce distinct subcellular translocations of these nPKCs in yeast cells. With this goal, immunofluorescence studies were carried out with yeast cells expressing nPKC-δ or -ε treated with 1 μM PMA, 1 μM coleon U or DMSO only. Since the results obtained with PKC-ε were not significantly different from those obtained with PKC-δ, the PKC-δ results were used as representative of the subcellular distribution obtained with the two nPKCs (Fig. 9A and B). The results showed that in the absence of activators, nPKCs were predominantly located at the cytosol of yeast cells (Fig. 9A and B; DMSO). In agreement with that reported for mammalian cells [28], PMA caused a marked translocation of nPKCs to the yeast plasma membrane (Fig. 9A and B; PMA). On the other hand, coleon U caused a pronounced translocation of nPKCs to the nucleus of yeast cells (Fig. 9A and B; coleon U).
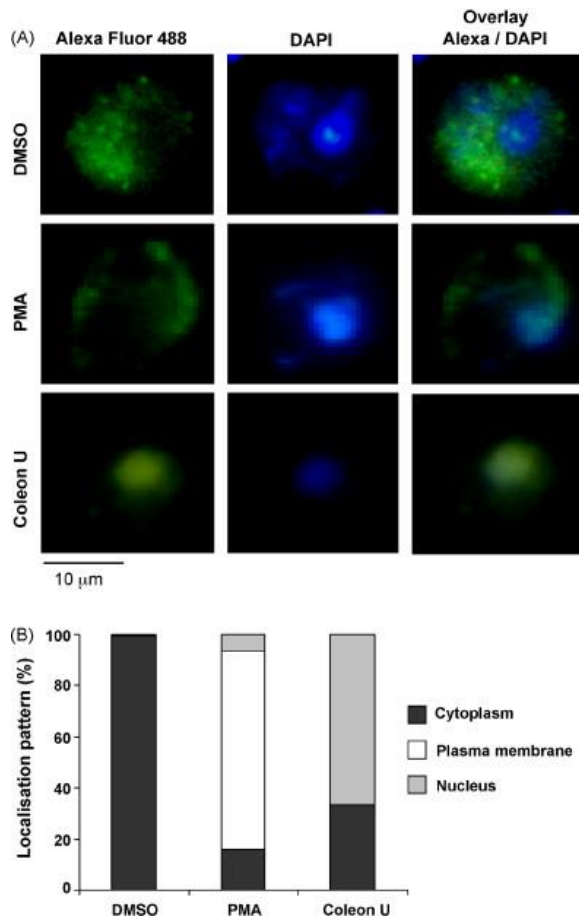
Fig. 9. Effect of PMA and coleon U on yeast subcellular localisation of PKC-δ. Analysis of PKC-δ localisation in yeast was carried out by immunofluorescence microscopy. (A) Cytoplasmic localisation of PKC-δ observed when yeasts were incubated with DMSO only; localisation of PKC-δ at the plasma membrane observed when yeasts were incubated with 1 μM PMA; nuclear localisation of PKC-δ observed when yeasts were incubated with 1 μM coleon U. (B) Quantification of subcellular distribution of PKC-δ in yeast cells incubated with PMA, coleon U or DMSO only; values correspond to the mean of three independent experiments.

Together, the results obtained showed that PMA and coleon U induced translocation of nPKCs to distinct yeast subcellular compartments.

4. Discussion

Previous studies from our group exploited the yeast PKC expression system not only in searching for isoform-selective PKC modulators [18–21], but also in studying the role of several PKC isoforms in apoptosis regulation [17]. In the present work, this yeast PKC assay was used to study the modulatory activity of coleon U on several PKC isoforms of the three PKC subfamilies, cPKCs (α and βI), nPKCs (δ and ε) and aPKC (ζ), considered as the most relevant isoforms in carcinogenesis. With this cell system, it was possible to analyse the effect of coleon U on each PKC isoform expressed in the same cellular background and without the genetic complexity of mammalian pathways and interference from other PKC family isoforms.

In agreement with that reported for human cells [1,2], we observed that 0.1–1 µM coleon U also induced yeast growth inhibition but only in cells expressing the nPKC-δ or -ε. This isoform-selectivity was further observed for 0.3 µM coleon U using an *in vitro* PKC assay. Together, these results led us to discover that coleon U is a selective activator of nPKC-δ and -ε. This work also studied the molecular mechanisms of action underlying coleon U-induced growth inhibition in yeast expressing nPKC-δ or -ε. We demonstrated that this coleon U antiproliferative effect was not linked to the induction of cell cycle arrest, but instead to the activation of an apoptotic cell death. This is in agreement with a previous study performed in lymphocyte cells, where detection of PS externalisation by annexin V allowed the antiproliferative effect of coleon U to be associated with apoptosis induction [2]. Herein, we have further shown that this coleon U-induced apoptosis involved the activation of a metacaspase- and mitochondrial-dependent pathway in yeast expressing nPKC-δ or -ε, as revealed by the increase in mitochondrial dysfunctions such as fragmentation, depolarization and ROS accumulation and by the complete abolishment of coleon U cytotoxic effect by the mitochondrial anti-apoptotic protein Bcl-xL.

Another relevant point from this work is the corroboration that a specific PKC isoform can induce, in the same cellular background, different cellular responses depending on the stimulus applied. In fact, comparing the effects of PMA and coleon U, we detected that these two PKC activators induced different effects in yeast due to the activation of the same PKC isoform. While PMA caused a G2/M cell cycle arrest, coleon U induced an apoptotic cell death in yeast expressing nPKC-δ or -ε. This can be explained by the ability of different stimuli to selectively translocate a PKC isoform to distinct subcellular compartments. In fact, it is believed that translocation of a specific PKC isoform to a cellular compartment is the major determinant of its specificity and function [28]. Indeed, similarly to results from mammalian cells [28,29], we found that in the absence of activators both nPKC-δ and -ε were localised in the cytosol of yeast cells. Additionally, as reported for PKC-δ[28,29] and PKC-ε[29] in mammalian cells, treatment of yeast cells with PMA induced translocation of these isoforms to the plasma membrane. On the other hand, coleon U treatment stimulated translocation of nPKC-δ and -ε to the nucleus of yeast cells. In accordance with the results obtained in yeast, recent studies performed with mammalian cells revealed that several pro-apoptotic kinases, specifically PKC-δ, undergo cytoplasmic-nuclear shuttling in response to DNA damage [30–32]. These studies demonstrated that upon exposure to a genotoxic stress, such as etoposide, PKC-δ accumulates in the nucleus. Whereas retention of PKC-δ in the cytoplasm is compatible with cell survival, its nuclear retention is required for commitment to apoptosis, showing that cellular localisation of PKC-δ regulates the survival/death pathway [30–32]. Indeed, nuclear targeting of kinases such as PKC-δ is considered a new and essential regulatory mechanism that directly influences the induction of apoptosis [32]. Thus, while modulation of nPKC-δ and -ε nuclear translocation by coleon U remains an open issue that deserves further studies, we corroborated previous data from mammalian cells showing that the correct cellular localisation is critical to the function of these two kinases.

Though several evidences supporting the involvement of mitochondria in coleon U-induced apoptosis are presented herein, we could not demonstrate mitochondrial accumulation of nPKC-δ or -ε after coleon U treatment. Interestingly, other authors demonstrated the involvement of mitochondria in PKC-δ-induced apoptosis in a rat vascular smooth muscle cell line based on the decrease of apoptosis by Bcl-2 and loss of $\Delta\psi_m$ without detection of PKC-δ mitochondrial localisation [33]. Another work also showed that although induction of apoptosis *via* activation of PKC-δ and -ε increased cytochrome *c* release and accumulation of ROS, no PKC-δ and -ε were detected in

mitochondria [8]. Supporting these data, we showed that also in yeast, under apoptotic conditions, PKC-δ and -ε seem to activate a mitochondrial pathway without directly interfering with this organelle. As stressed by Yoshida [32], the molecular devices that allow various stimuli, such as coleon U, to be transmitted from the nucleus into the mitochondrion and which represents a point of integration for the different apoptotic signalling cascades are still unclear. Further studies are underway in yeast to clarify this issue.

In conclusion, this work identifies a new isoform-selective small-molecule with potential pharmacological applications. Indeed, as a potent and selective activator of the nPKC-δ and -ε, coleon U represents a promising tool to further understand the nPKC-δ and -ε cellular signalling pathways in mammalian cells. Furthermore, the absence of an effect on cPKC-α and -β and aPKC-ζ, reported as anti-apoptotic proteins, and its selectivity to induce an apoptotic pathway dependent on nPKC-δ and -ε activation, indicate that coleon U is a promising compound for evaluation as an anti-cancer drug. This work also shows, for the first time, that the yeast PKC expression system allows reconstituting distinct subcellular translocations of a specific PKC isoform and the subsequent different cellular responses previously reported for mammalian systems. Considering these similarities and the advantages provided by the genetic tractability of yeast, a broader outcome of this study is the validation of this cell model to unravel the intra-organelle communication systems and their roles in the PKC isoform apoptotic signalling network, as well as to study the molecular mechanisms of action of compounds with potential therapeutic applications.

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