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Evidence in favour of a role for peripheral-type benzodiazepine receptor ligands in amplification of neuronal apoptosis

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The mitochondrial peripheral benzodiazepine receptor (PBR) is involved in a functional structure designated as the mitochondrial permeability transition (MPT) pore, which controls apoptosis. PBR expression in nervous system has been reported in glial and immune cells. We now show expression of both PBR mRNA and protein, and the appearance of binding of a synthetic ligand fluo-FGIN-1-27 in mitochondria of rat cerebellar granule cells (CGCs). Additionally, the effect of PBR ligands on colchicine-induced apoptosis was investigated. Colchicine-induced neurotoxicity in CGCs was measured at 24 h. We show that, *in vitro*, PBR ligands 1-(2-chlorophenyl-N-methylpropyl)-3-isoquinolinecarboxamide (PK11195), 7-chloro-5-(4-chlorophenyl)-1,3-dihydro-1-methyl-2H-1,4-benzodiazepin-2-one (Ro5-4864) and diazepam (25–50 μM) enhanced apoptosis induced by colchicine, as demonstrated by viability experiments, flow cytometry and nuclear chromatin condensation. Enhancement of colchicine-induced apoptosis was characterized by an increase in mitochondrial release of cytochrome c and AIF proteins and an enhanced activation of caspase-3, suggesting mitochondrion dependent mechanism that is involved in apoptotic process. Our results indicate that exposure of neural cells to PBR ligands generates an amplification of apoptotic process induced by colchicine and that the MPT pore may be involved in this process.

Keywords: AIF; cerebellar granule cells; colchicine; Cyt c; mitochondrial permeability transition; peripheral benzodiazepine receptor.

Introduction

The peripheral-type benzodiazepine receptor (PBR), so named as a result of its affinity for the benzodiazepine diazepam, is a ubiquitous protein found in most steroidogenic tissues. PBR is located primarily in the outer mitochondrial membrane and is associated with the voltage-dependent anion channel protein.[1] It is involved in the regulation of cholesterol transport from the outer to the inner mitochondrial membrane, heme biosynthesis, anion transport, immunomodulation and also in the regulation of cell proliferation. It is part of the mitochondrial permeability transition (MPT) pore,[2] a multiprotein complex located at the contact site between the inner and outer mitochondrial membranes that is intimately involved in the initiation and regulation of apoptosis in various cells, including hepatocytes,[3] fibroblasts[4,5] and thymocytes;[6,7] it is also involved in several oncogenic processes.[8–10]

In the central nervous system (CNS), PBR is located primarily in glial cells,[11] and has been used as a quantitative marker in several studies of CNS injury, including inflammation,[12] metabolic stress,[13] and traumatic, ischemic[12,14,15] or chemically-induced brain injury.[16–18] In these studies the loss of neurons was accompanied by an increase in PBR density. Increased expression of PBR after neuronal injury occurs primarily in microglia/macrophages rather than astrocytes.[15,19] In these cells, PBR ligands can promote survival by favouring glial cell proliferation and/or promoting the production of mediators such as neurosteroids, cytokines or other neurotrophic factors that support neuronal survival. On the other hand, PBR ligands, 1-(2-chlorophenyl-N-methylpropyl)-3-isoquinolinecarboxamide (PK11195) or 7-chloro-5-(4-chlorophenyl)-1,3-dihydro-1-methyl-2H-1,4-benzodiazepin-2-one (Ro5-4864), have been shown to inhibit proliferation of cultured astrocytes,[20] lymphoid cells and macrophages.[21] Similar changes in PBR density are observed in acute and chronic neurodegenerative states in human, *e.g.* in temporal cortex obtained from patients with Alzheimer's disease.[22,23] A highly significant increase in PBR density was also observed in the putamen, and a moderate but significant

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increase in the frontal cortex of patients suffering from Huntington's disease.[24]

PBR and other pro-apoptotic proteins expression in lymphocytes and platelets, have been extensively used to demonstrate that some of the changes observed at the SNC—increased oxidative stress, for example—can also be observed peripherally.[25–27] In this way, recently, it was found clear modifications in the levels of PBR protein involved in lymphocytes of Parkinson disease patients.[28]

PBR ligands by their interaction with the MPT pore, also enhance the susceptibility of cells to induction of apoptosis by a variety of different stimuli, including DNA damage, glucocorticoid receptor binding and ceramide, and can reverse the suppression of apoptosis by Bcl-2.[6,9,29] Thus, it has been suggested that, collapse of the mitochondrial membrane potential (Ψm) could modulate the apoptotic process. Alterations in mitochondrial structure and function play a crucial role in apoptosis by releasing apoptotic factors from mitochondria including Cyt c and apoptosis-inducing factor (AIF).[30,31]

The localization of PBR has been described in some neuronal cell types, such as cells of the olfactory bulb,[32,33] neuroblastoma cells,[9] cultured cortical neurons[34] and rat sensory neurons.[35] Little is known about the role of PBR in neurons, but several reports indicate that PK11195 and protoporphirin IX are able to increase reactive oxygen species (ROS) in cultured neurons.[34] Furthermore, PBR ligands have been shown to elicit multiple responses in cultured neurons,[36–38] and to stimulate neuronal firing.[39,40]

Disassembly of microtubules and cytoskeletal pathology are known to play a major role in many neurological disorders, including Alzheimer's, Parkinson's and Huntington's diseases, consequently, colchicine is a widely used tool in *in vitro* models of this type of neurodegeneration.[41–43]

The first goal of this work was to describe the mitochondrial localization of PBR in primary cultures of cerebellar granule cells (CGCs). The second aim, because the PBR is an important component of the MPT, was to examine the effect of benzodiazepine receptor ligands on CGCs and to evaluate whether these compounds modulate the apoptotic effects of colchicine, a well-known neurotoxin that induces apoptosis through the activation of the mitochondrial apoptotic pathway.

Materials and methods

Characterization of PBR in CGCs

Cerebellar granule cell cultures. Primary cultures of CGCs were prepared from 7-day-old Sprague Dawley rat pups according to Verdaguer *et al.*[44] Briefly, cerebella were quickly removed and cleaned of meninges, manually sliced with a sterile blade, dissociated with trypsin and treated with DNase. Cells were adjusted to 8×10^5 cells/ml and plated on poly-L-lysine-coated culture plates at a density of 3.2×10^5 cells/cm^2. Cultures were grown in basal medium with Eagle's salts (BME) containing 10% FCS, 2 mM L-glutamine, 0.1 mg/ml gentamicin and 25 mM KCl. Cytosine arabinoside (10 μM) was added 16–18 h after plating, in order to inhibit the growth of non-neuronal cells. Cultures prepared using this method were more than 95% enriched in granule neurons, as assessed by GFAP immunocytochemistry (data not shown).

PBR immunodetection. Immunocytochemistry was used to analyze PBR presence in CGCs. Cells were grown on sterile glass coverslips, washed twice in phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde/PBS, pH 7.4 for 1 h at room temperature. Then CGCs were preincubated at room temperature for 30 min in PBS containing 0.3% Triton X-100 and 30% normal horse serum at room temperature. After blocking, cells were incubated overnight at 4°C with a polyclonal antibody against PBR (1:500; R&D Signalling) and a monoclonal antibody against GRP75, mitochondrial marker (1:1000; Stressgen). Cells were then washed extensively and incubated for 1 h at room temperature with secondary antibody (FITC-conjugated anti-rabbit and rhodamine-conjugated anti-mouse, both at a dilution 1:100; Sigma). Coverslips were thoroughly washed and mounted in Mowiol® 4–88. Labeling was analyzed by fluorescence microscopy at 100× magnification (Eclipse TE 200, Nikon Instech, Corp., Japan) and their images captured digitally. Western blot analysis was performed on aliquots of treated cells containing 30 μg protein; samples were separated by electrophoresis on 10% acrylamide, and polyclonal antibody against PBR (1:2000; R&D Signalling) was used to immunodetect specific bands, following the method described below for α-spectrin breakdown (see Assay of caspase-3 enzymatic activity).

Fluorescent localization of PBR. After 7–10 days *in vitro*, cells were washed twice with sterile phosphate-buffered saline (PBS) and incubated for 45 min with 1 μM fluo-FGIN-1-27 (NBD FGIN-1-27, Alexis) with or without the indicated competing ligands PK11195 and FGIN-1-27 [(N,N-Di-hexil-2-(4-fluorophenyl)indole-3-acetamide, Alexis] at 100 μM. At the end of the incubation period, the coverslips were washed with PBS and examined by fluorescence microscopy using a Nikon Eclipse fluorescence microscope. The 45 min incubation time was established based on preliminary works that indicated that visibility of intracellular fluorescence after a 20 min incubation and reached saturation at around 40 min. The concentration of fluo-FGIN-1-27 used was also determined based on preliminary dose-response studies. These experiments showed that intracellular fluorescent labeling could

be seen using 10 and 100 nM fluo-FGIN-1-27 but that a concentration of 1 μM fluo-FGIN-1-27 was required to obtain a consistent and reproducible labeling. It is important to note that the fluorescent binding studies were performed directly on living cells in culture, in contrast to other fluorescence studies that were performed on PFA-fixed cells that were stored at 4°C, prior to immunocytochemical analysis.

Reverse transcriptase-PCR. Total RNA was extracted from CGCs using Tri Reagent from Sigma (Saint Louis, USA). First strand cDNA was reverse transcribed from 2 μg of total RNA using a First-Strand Synthesis System kit from Invitrogen Corporation (Carlsbad, USA). The same amounts of cDNA were subsequently used for PCR amplification (32 cycles; 95°C for 1 min, 59°C for 1 min and 72°C for 2 min each cycle). The expression of β-actin mRNA was used as a standard. The rat-specific PBR PCR primers (F: 5′-ATGGGAGCCTACTTTGTGCGT-3' and R: 5′-CCATACCCCATGGCCGAATAC-3′) were used to generate a PBR PCR product of 127 bp. β-actin primers (F: 5′-TTGTAACCAACTGGGACGATATGG-3′ and R: 5′-GATCTTGATCTTCATGGTGCTAGG-3′) were used to generate a fragment of 700 bp. The PCR products were separated on a 2% agarose gel. Electrophoresis bands were quantified by densitometry.

Functional relevance of PBR ligands in colchicine-induced neurotoxicity

Treatment of CGCs and survival assay. CGCs were used after 7–10 days *in vitro*. Colchicine was dissolved in complete culture medium and added to the cell culture (1 μM final concentration). To investigate the effect of PK11195, Ro5-4864, diazepam and clonazepam, they were added to the medium at the specific concentrations (10 to 50 μM) 30 min before addition of the neurotoxin. Cell death was determined 24 h after neurotoxin addition using the MTT assay as described below.

Assessment of neuronal injury. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tretrazolium bromide) was added to the cells at a final concentration of 250 μM and incubated for 1 h to allow the reduction of MTT to produce a dark blue formazan product (Hansen *et al*., 1989). The medium was removed, and cells were dissolved in dimethylsulfoxide. Formazan formation was assayed by measuring the absorbance change (595 nm) on a microplate reader (Bio-Rad Laboratories, CA, USA). Viability results are expressed as percentages of the absorbance measured in non-treated cells.

Analysis of apoptosis rate by flow cytometry and fluorescence microscopy. Apoptosis was measured 24 h after colchicine addition. Briefly, propidium iodide (PI, 10 μg/ml) and 0.1% Triton-X100 was added to the culture medium 30 min before cytofluorometic analysis. Cells were collected from culture plates by pipetting. Flow cytometry experiments were carried out using an Epics XL flow cytometer (Coulter Corp. Hialeah, FL), as described previously.[45] Red fluorescence was projected on a 1024 monoparametrical histogram.

PI staining was used to detect morphological evidence of apoptosis. CGCs were grown on glass coverslips and, after treatment with colchicine (1 μM), were fixed in PFA/PBS, pH 7.4 for 1 h at room temperature. After washing with PBS, they were incubated for 3 min with a solution of PI in PBS (10 μg/ml). Coverslips were mounted in Mowiol® 4–88. Stained cells were visualized under UV illumination using the 20× objective (Nikon Eclipse) and their digital images were captured.

The apoptotic cells exhibited shrunken, brightly fluorescent, apoptotic nuclei showing high fluorescence and condensed chromatin compared with non-apoptotic cells. Apoptotic cells were scored by counting at least 500 cells from six different fields for each sample in three different experiments.

Assay of caspase-3 enzymatic activity. We used the colorimetric substrate Ac-DEVD-p-nitroaniline (Sigma) for the determination of caspase-3 activity according to the following method. 24 h after treatment with 1 μM colchicine, CGCs were collected in a lysis buffer (50 mM Hepes, 100 mM NaCl, 0.1% CHAPS, 0.1 mM EDTA, pH 7.4). 0.25 μg/μl of protein was incubated with 200 μM Ac-DEVD-p-nitroaniline in assay buffer (50 mM HEPES, 100 mM NaCl, 0.1% CHAPS, 10 mM dithiothreitol, 0.1 mM EDTA, pH 7.4) in 96-well plates at 37°C for 24 h. Absorbance of the cleaved product was measured at 405 nm in a microplate reader (Bio-Rad). Results were expressed as percentages of the absorbance measured in vehicle-treated cells.

To assess α-spectrin breakdown as a measure of caspase-3 activity,[46] Western blot analysis was performed on aliquots of treated or control cells, containing 5 μg of protein per sample. Briefly, samples were placed in sample buffer (0.5 M Tris-HCl pH 6.8, 10% glycerol, 2% (w/v) SDS, 5% 2-β-mercaptoethanol, 0.05% bromophenol blue) and denatured by boiling at 95–100°C for 5 min. Samples were separated by electrophoresis on 5% acrylamide gels. Thereafter, proteins were transferred to polyvinylidene fluoride (PVDF) sheets (Immobilon™-P, Millipore Corp., Bedford, MA) using a transblot apparatus (Bio-Rad). Membranes were blocked overnight with 5% non-fat milk dissolved in TBS-T buffer (50 mM Tris, 1.5% NaCl, 0.05% Tween 20, pH 7.5). They were then incubated with a primary antibody against α-spectrin (1:1000; Chemicon Int. After 90 min, blots were washed thoroughly in TBS-T buffer and incubated for 1 h with a peroxidase-conjugated IgG antibody (Amersham

Corp., Arlington Heights, IL). Immunoreactive protein was visualized using a chemiluminescence-based detection kit according to the manufacturer's protocol (ECL kit, Amersham Corp.). Routinely, protein load was monitored using phenol red staining of the blot membrane. Changes in the optical density (OD) ratio between the 129 kD and 240 kD bands was taken as an index of caspase-3 activation.

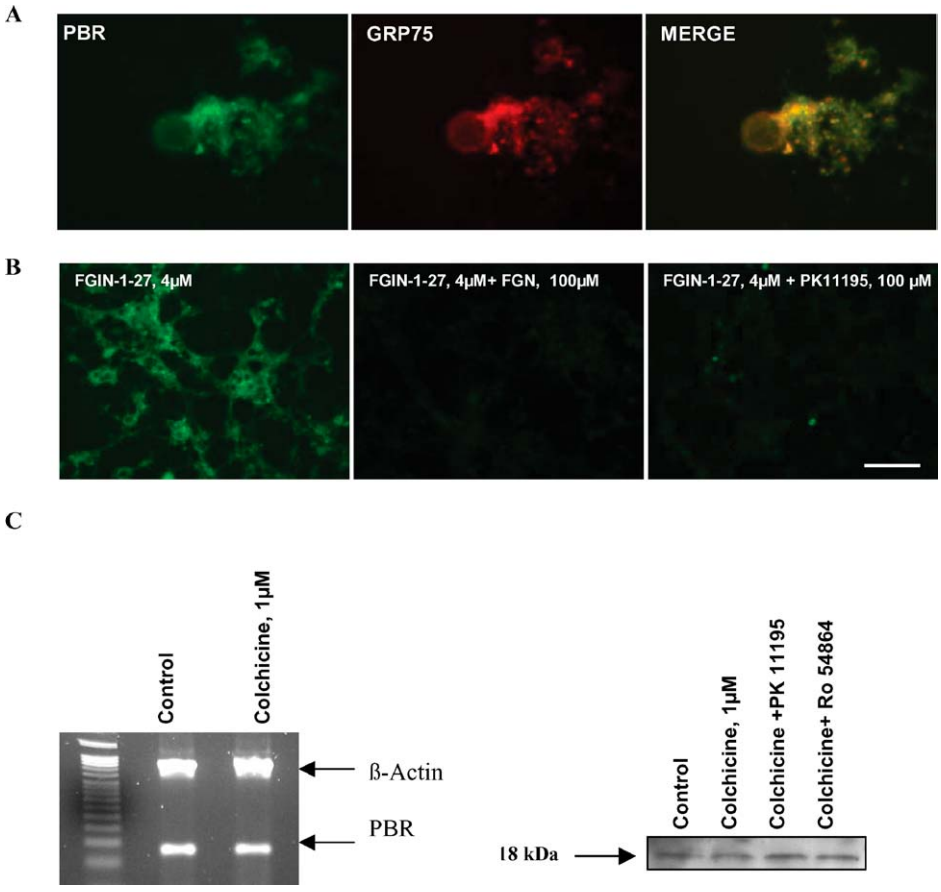
Immunocytochemistry against AIF and Cyt c. CGCs were grown on sterile glass coverslips. After stimuli, cells were washed twice in PBS and fixed in 4% paraformaldehyde/PBS, pH 7.4 for 1h at room temperature and then permeabilized in 0.3% Triton X-100 for 10 min. After blocking with goat serum (1 h at room temperature), cells were incubated overnight at 4°C with mouse anti-Cyt c (1:500; Neomarkers) or anti-AIF (1:500; Santa Cruz). Cells were then washed extensively and incubated for 1 h at room temperature with secondary antibody (Rho-conjugated anti-mouse, 1:100; Sigma). Co-localization with mitochondria was assessed by pre-incubating cells with 0.5 μM Mito Tracker Red (CM-H_2XROS®, Molecular Probes, Inc.) for 1 h prior to fixation. Coverslips were thoroughly washed and mounted in Mowiol®4-88 and labeling was analyzed using a fluorescence microscope at 100× magnification (Nikon Eclipse).

Statistics. The data are presented as the mean ± SEM. For statistical comparisons, one-way analysis of variance followed by Tukey's test was used for multiple comparisons or Student's t test for pairs of data. Values were accepted as statistically significant when $p < 0.05$.

Results

Evidence of PBR localization in CGCs. We evidenced the PBR using the specific fluorescent ligand fluo-FGIN-1-27. Fluorescent images (Figure 1A) indicate that the labeling was notably cytoplasmic.

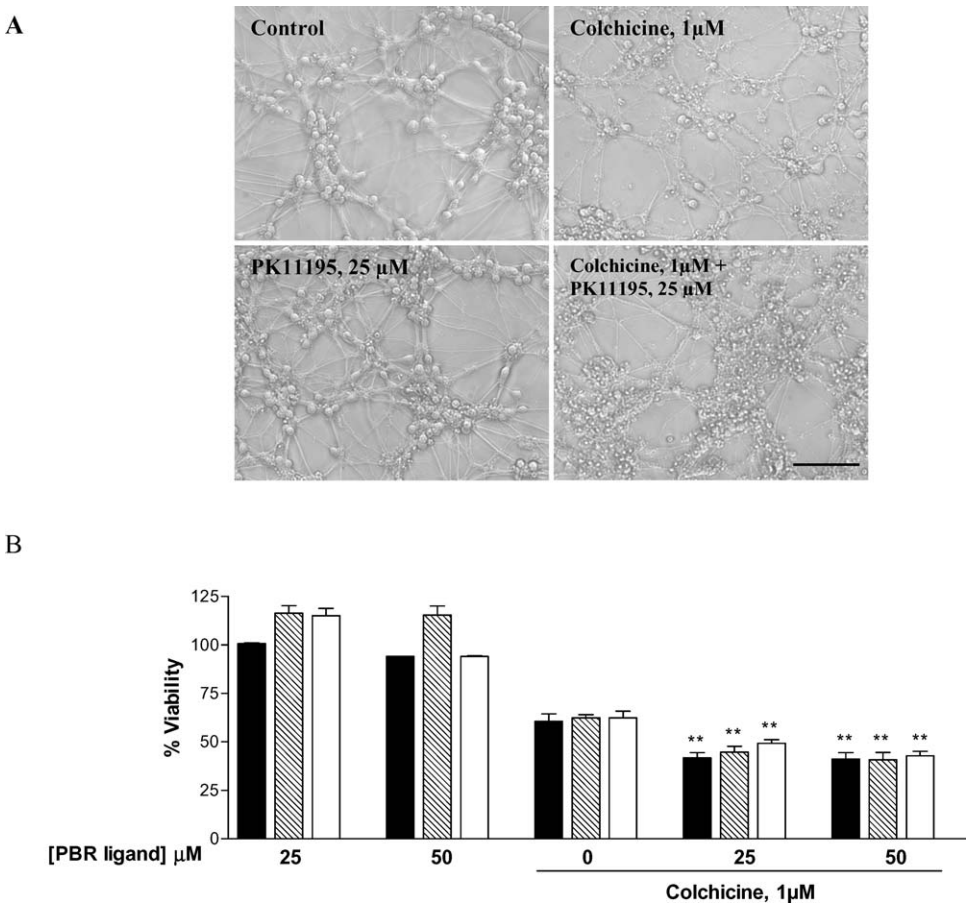
Figure 1. (A) Immunocytochemistry against PBR (green) in CGCs. Co-localization with mitochondria was analysed by GRP75 inmunostaining (red). 100× lens. (B) Fluorescent staining with fluo-FGIN-1-27 in the absence or presence of FGIN-1-27 or PK11195. 20× lens. Similar results were obtained in three different experiments. Calibration bar, 10 μm. (C) Representative RT-PCR and Western blot analysis showing the transcriptional activity for PBR and PBR protein obtained from CGCs. ß-actin was used as a control gene in PCR experiments, and phenol red staining as control of protein loading in WB experiments



Fluorescent staining was completely displaced by a 100-fold excess of the isoquinoline PK11195 and the 2-aryl-3-indole-acetamide prototype FGIN-1-27, further demonstrating the specificity of the interaction of fluo-FGIN-1-27 with the PBR. The possibility that some of the fluorescent labeling seen may be due to non-specific trapping of the compound in the lysosomal fraction of the cells should be also considered. Taking into account the subcellular fractionation, immunocytochemical studies were performed to confirm the mitochondrial localization of PBR in CGCs using a PBR-specific antibody. The presence of punctate cytoplasmic labeling suggests a mitochondrial localization of the receptor, and this was confirmed by double inmunocytochemistry with GRP75, a protein that localizes into the mitochondria and is widely used as a mitochondrial marker (Figure 1B). In addition, we detected PBR gene transcription by RT-PCR and PBR expression by Western blot. The results revealed that neither transcription nor protein changed after addition of colchicine or colchicine plus PBR ligands (Figure 1C).

Viability studies after treatment of CGCs with colchicine or colchicine plus PBR ligands. Our first goal was to corroborate the neurotoxicity of 1 μM colchicine in CGCs that we demonstrate previously[41,47] by MTT assays. Positive results with this method are indicative of mitochondrial membrane disruption and of increased cell death. When cells were pre-treated with PK 11195, Ro5-4864 or diazepam, colchicine-induced neurotoxicity was significantly increased (Figure 2); this was not observed in clonazepam pre-treated CGCs, indicating that the potentiation is independent of central benzodiazepine receptor. We also evaluated the neurotoxic effect of the PBR ligands on CGCs in the absence of colchicine, at doses ranging from 1 μM to 100 μM. At these

Figure 2. (A) Representative phase-contrast images of CGCs cultures in the presence of several drug treatments Scale bar, 10 μM. (B) Viability studies in CGCs treated for 24 h with 1 μM colchicine in the absence or the presence of PK 11195 (black bars), Ro5-4864 (dashed bars) and diazepam (open bars). Data are shown as mean ± SEM of the percentage change versus control cells, arbitrarily set at 100%. The statistical analysis was carried out using one-way ANOVA followed by Tukey's test. ** $p < 0.01$ vs. colchicine values. (calibration bar, 10 mm)



concentrations we did not observe any toxic effect in terms of either viability or cell morphology (Figure 2A–B).

PBR ligands increase colchicine-induced apoptosis in CGCs. Flow cytometric studies demonstrated that colchicine increased the percentage of apoptotic nuclei. Pre-treatment with ligands PK11195, Ro5-4864 and diazepam, but not with clonazepam, resulted in a significantly higher number of apoptotic cells. However, PBR ligands alone did not induce apoptosis compared with control values (Figure 3).

Nuclei of paraformaldehyde-fixed CGCs were viewed by PI staining, a reliable method for the evaluation of neuronal nuclear morphology. A distinguishing feature of apoptosis is the condensation and fragmentation of nuclear chromatin. The nuclear chromatin of control CGCs permeabilized and stained with PI was dimly

Figure 3. Measurements of the fraction of apoptotic cells (percentage of hypodiploid cells measured by cytometric analysis) after 1 μM colchicine-induced apoptosis in the absence or the presence of PK 11195 (A), Ro5-4864 (B) or diazepam (C). Effects of z-VAD.fmk on neurotoxicity induced by colchicine plus PBR ligands were quantified for each experimental condition. Each point represents the mean ± SEM of 4 wells from 3 to 5 cultures. The statistical analysis was carried out using one-way ANOVA followed by Tukey's test. $^{***}p < 0.001$ vs. control values; $^{\#}p < 0.05$ $^{\#\#}p < 0.01$ vs. colchicine values; $^{\$\$\$}p < 0.001$ vs. colchicine plus PBR ligands values.

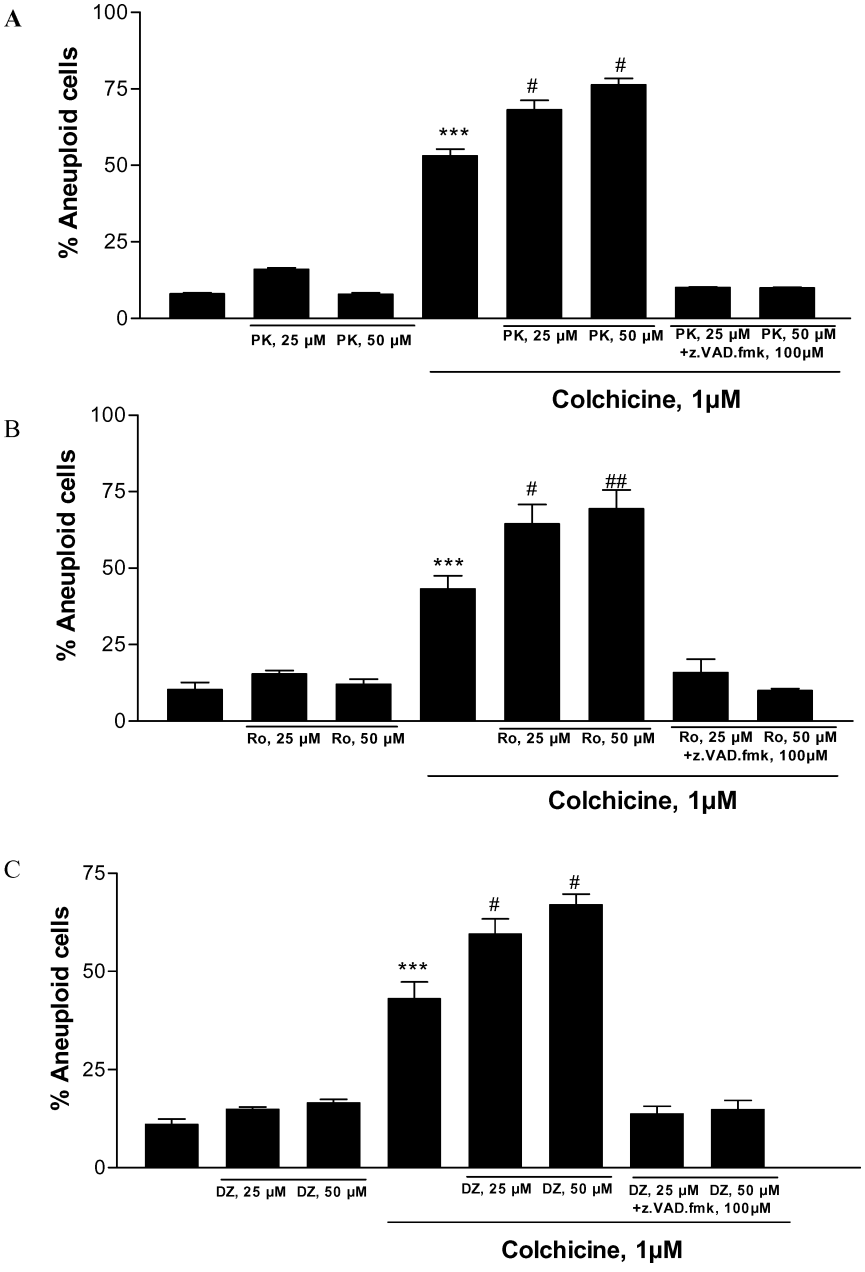
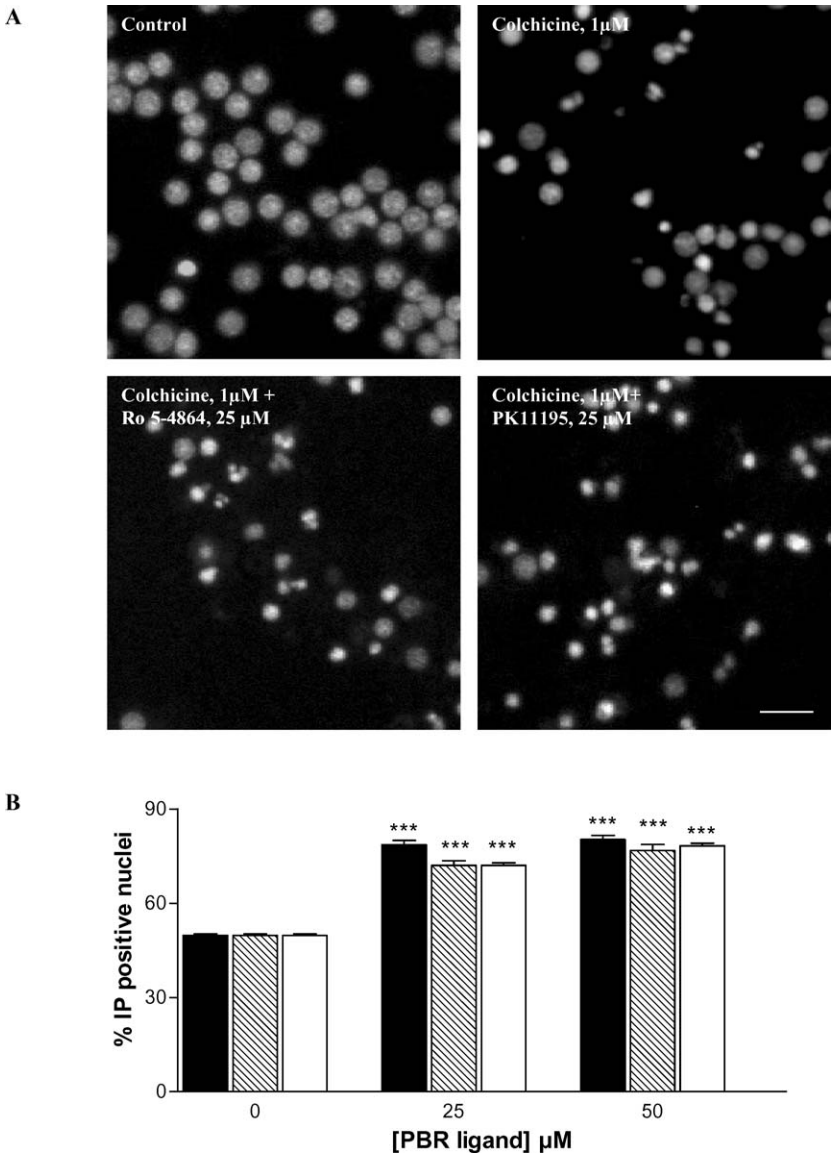


Figure 4. (A) Representative microscopy fields showing fluorescence labeling of DNA in the nuclei of control CGCs, CGCs treated with 1 μM colchicine and CGCs pre-treated PK11195 and Ro5-4864 prior to colchicine treatment. Calibration bar, 10 μm. (B) Quantitative evaluation of 1 μM colchicine-induced apoptosis and the effect of 25 μM or 50 μM PK11195 (black bars), Ro5-4864 (dashed bars) or diazepam (open bars) using PI staining. Results are expressed as the percentage of PI positive nuclei. Values were obtained from counts of at least 500 nuclei in four or more random microscope fields per condition and error bars represent the SEM of four experiments. The statistical analysis was carried out using one-way ANOVA followed by Tukey's test. *** $p < 0.001$ vs. colchicine values.



fluorescent, with uncondensed chromatin in large nuclei (10.25 $\pm$ 4.15, $n = 3$; Figure 4). 24 h colchicine treatment resulted in shrunken, brightly fluorescent, condensed chromatin-containing nuclei (49.76 $\pm$ 1.34, $n = 10$). The number of condensed chromatin-containing nuclei increased significantly in cultures treated with PBR ligands but not in those treated with clonazepam. Results with 50 μM PK11195, Ro-54864 and diazepam were: 80.29 $\pm$ 2.77%, 76.75 $\pm$ 3.88% and 78.25 $\pm$ 1.65% respectively ($p < 0.001$ vs colchicine-treated cells; Figure 4).

Caspase-3 activation induced by colchicine: Effect of PBR ligands. A dramatic increase (100%) in caspase 3 activity measured by the cleavage of chromogenic substrate was detected at 24 h. Neither 25 nor 50 μM PK11195, Ro-4864 or diazepam, added to cell culture 30 min before insult, increased caspase-3 activity, probably due to the lack of sensitivity of the biochemical method (Table 1). For this reason, α-spectrin cleavage was examined to achieve a more sensitive analysis of caspase-3 activation upon CGCs treatment with colchicine plus PBR ligands. Western blot analysis revealed that colchicine

Table 1. Spectrophotometric analysis of caspase-3 activity by measurement of the absorbance of a cleavage product at 405 nm in the different conditions tested. $p < 0.001$ vs. control values

Experimental conditions	Mean absorbance Arbitrary units	SEM	*n*
Control	0.18	0.02	7
Colchicine, 1 μM	0.51***	0.05	7
Colchicine + Diazepam (25 μM)	0.59	0.05	4
Colchicine + Ro5-4864 (25 μM)	0.54	0.07	4
Colchicine + PK11195 (25 μM)	0.49	0.03	4

induces cleavage of full-length α-spectrin to the caspase-generated 120 kDa fragment. Pretreatment of CGCs with 25 μM PK11195, Ro5-4864 or diazepam potentiated α-spectrin cleavage (Figure 5). To confirm the involvement of the caspase pathway, 100 μM z-VAD-fmk was added to cultures 24 h before colchicine treatment. Cytometric and morphological analysis of PI stained nuclei showed that this compound decreased the number of apoptotic cells by up to 75%, both in response to colchicine and colchicine plus PBR ligands, confirming an enhancement of the apoptotic pathway by PBR ligands in colchicine-treated cells (Figure 3).

PBR ligands potentiate the intrinsic apoptotic pathway induced by colchicine. We evaluated the effect of PBR ligands (25 μM) on the intrinsic pathway activated by colchicine treatment in CGCs. The subcellular distribution of several proteins involved in the intrinsic apoptotic pathway was examined by immunocytochemistry. First, AIF mitochondrial release was studied, revealing a pattern of AIF staining that was modified from a punctated mitochondrial pattern to a nuclear one after 6 and 24 h colchicine treatment; the level of staining was enhanced and this redistribution was augmented in cells pre-treated with PBR ligands (Figure 6).

Cyt c release from mitochondria to the cytosol was then studied. Cyt c was released after 6 h of colchicine treatment and the release was potentiated by PBR ligands (Figure 7). Cyt c staining after 24 h was similar in colchicine and colchicine plus PBR ligand-treated cells, co-localization with mitochondrial staining was made at 24 h. (Figure 8).

These results indicate that PBR ligands act favouring the aperture of MPT pore in CGCs facilitating the release of Cyt c and AIF and increasing the neurotoxic effects of colchicine.

Figure 5. 1 μM colchicine-induced increase in caspase-3 activity in CGCs and the effect of pre-treatment with 25 μM PBR ligands (PK, PK11195; Ro, Ro5-4864; DZ, diazepam). Results are shown as the mean ± SEM of 3 determinations from 3 different cultures. (A) Bar chart showing the semiquantitative 120 kD/280 kD ratio (O.D., arbitrary units) under the same experimental conditions. (B) Representative Western blot analysis of the α-spectrin degradation product [46] as an indicator of caspase-3 activity in CGCs treated with colchicine or colchicine plus PBR ligands. The statistical analysis was carried out using one-way ANOVA followed by Tukey's test. $^{**}p < 0.01$, $^{***}p < 0.001$ vs. control values; $^{\#}p < 0.05$ $^{\#\#\#}p < 0.001$ vs. colchicine values.

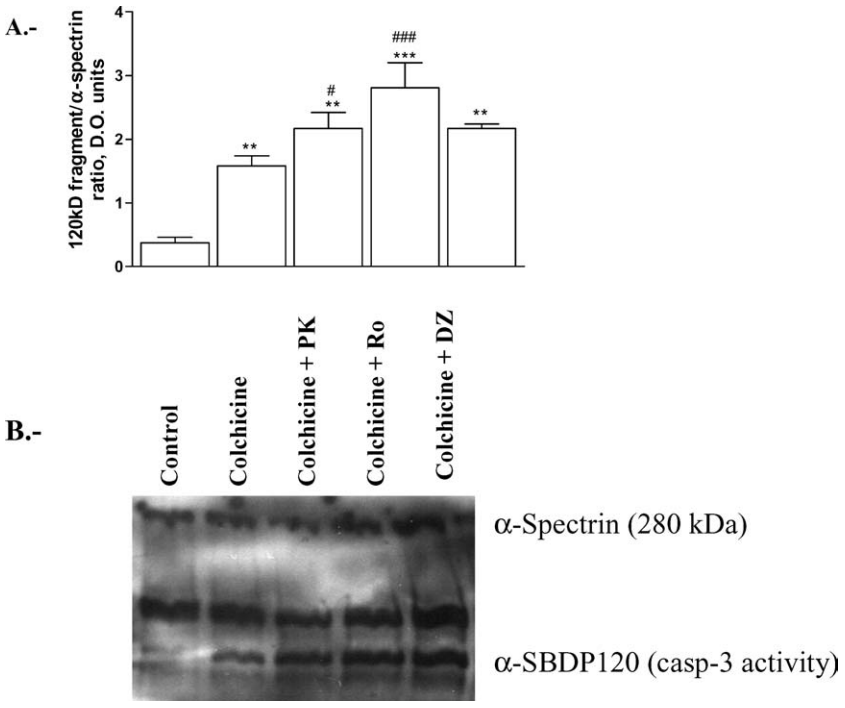


Figure 6. Representative images from immunofluorescence staining of AIF in CGCs after 6 and 24 h of colchicine-treatment in the presence or absence of PBR ligands. The results show AIF-positive nuclei at 6 and 24 h post-colchicine treatment when plates were treated with PBR ligands. Insert scale bar, 5 μm.

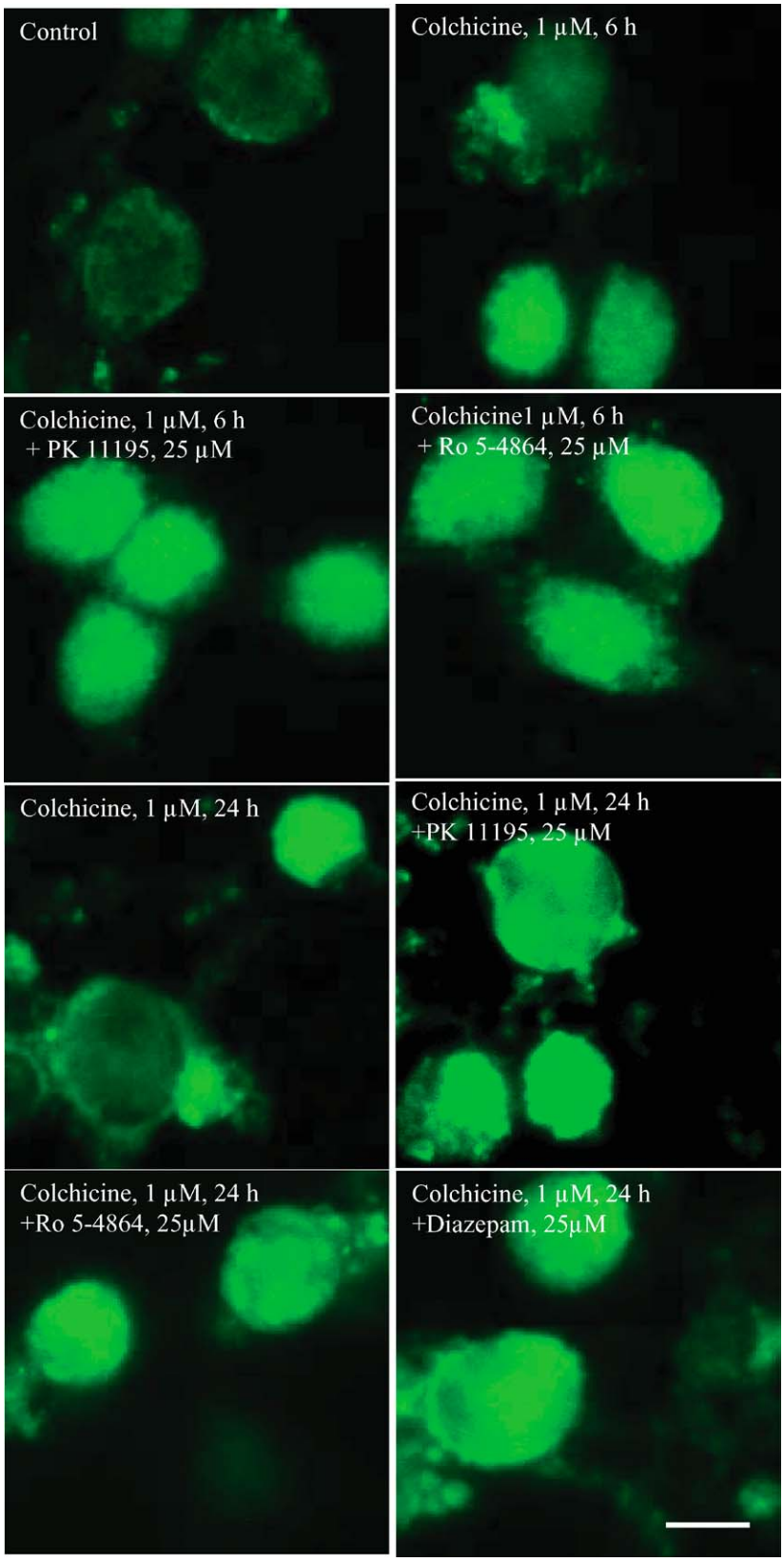
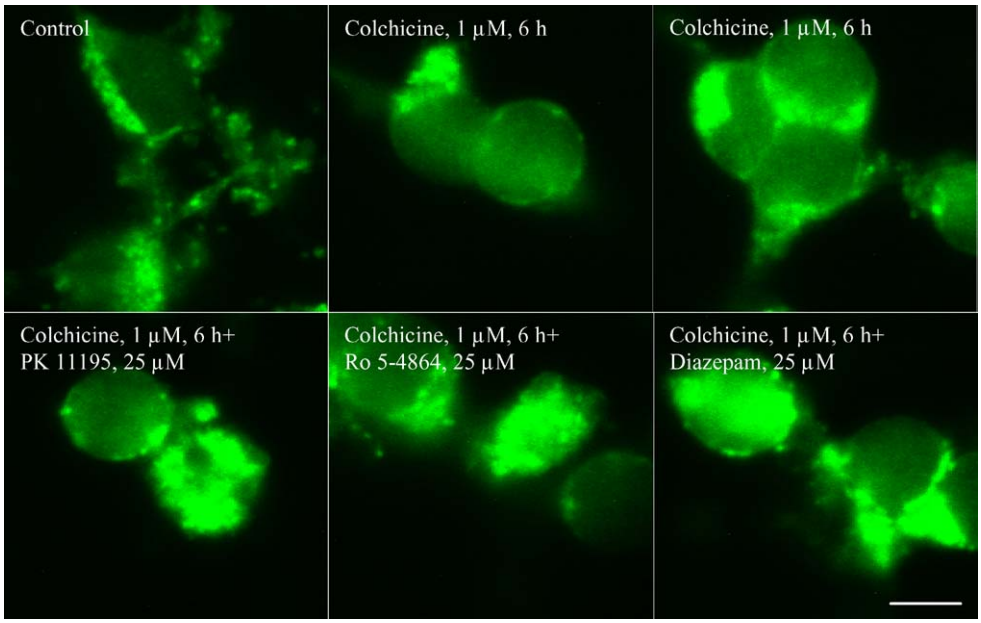


Figure 7. Potentiation of colchicine-induced Cyt c release in the presence of PBR ligands. The images show Cyt c release after 6 h colchicine-induced apoptosis in the absence or presence of PBR ligands. Insert scale bar, 5 μm.



Discussion

The presence of PBR in glial cells mitochondria is widely described,[11,48] but the neural localization of this receptor is less accepted. Nevertheless, PBR have been reported to be localized in the olfactory neurons,[32,33] neuroblastoma cells[9,49] and cortical neurons.[34]

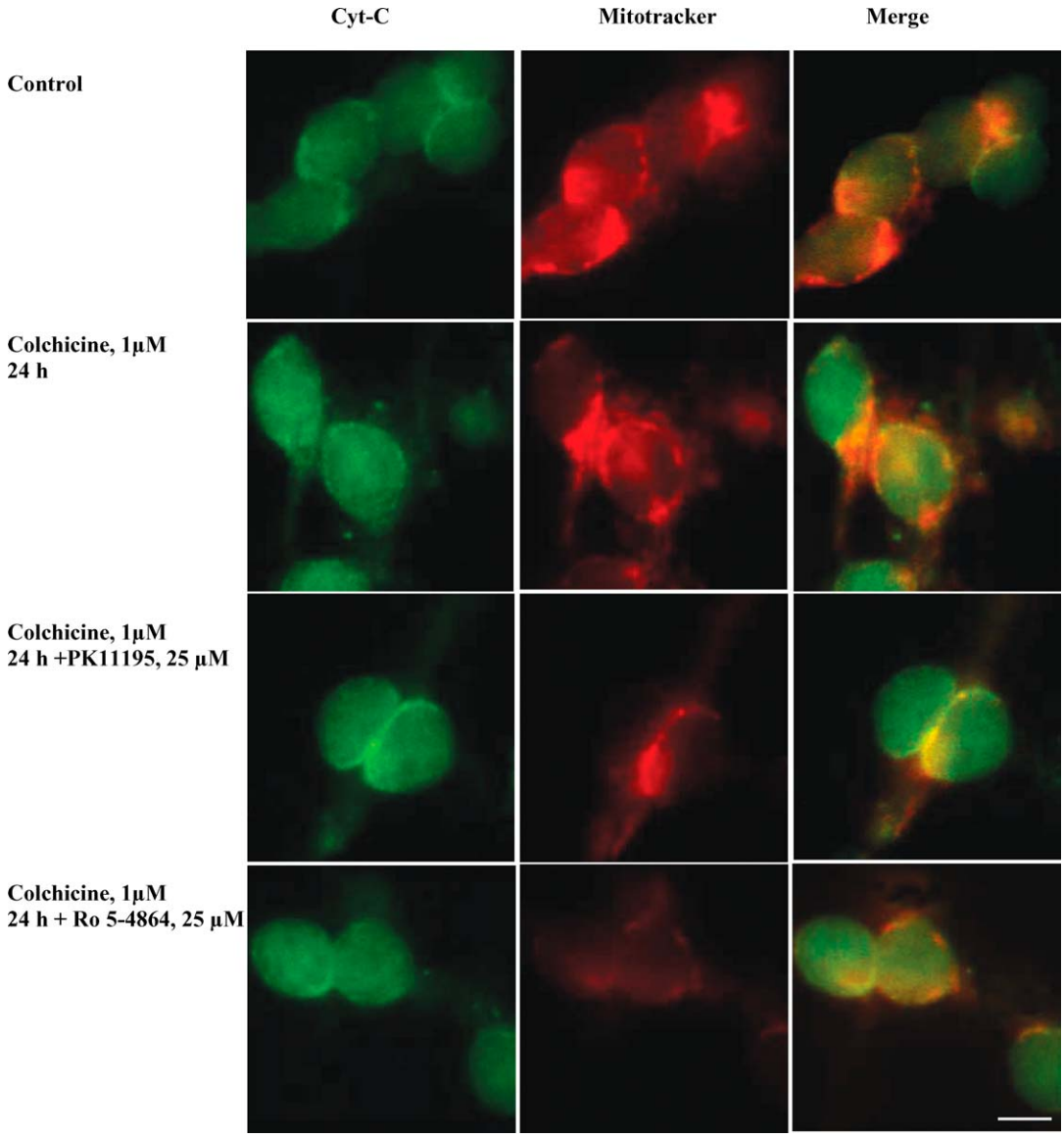
In this study, we have demonstrated the presence and mitochondrial localization of PBR in CGCs. Furthermore we have shown, in agreement with other reports, that PBR ligands enhance the pro-apoptotic effect of the microtubule disturbing drug colchicine. The potentiation of the neurotoxic effect of colchicine by PBR ligands in CGCs was at first surprising, as the presence of glial cells was very low in these primary cultures and the use of c-arabinoside prevented their proliferation. Intracellularly localized PBR were found in primary cultures of CGCs, using fluo-FGIN-1,27, an isoquinoline that binds to the 18 kDa PBR protein.[50,51] Moreover, immunocytochemical studies reveal that PBR is localized to mitochondria in neurons. We also detected the expression of PBR in CGCs by RT–PCR and Western blot. Taken together, these data clearly demonstrate the presence of PBR in cerebellar neurons.

In our study, PBR ligands enhanced colchicine-induced apoptosis, as measured by flow cytometry and morphological analysis of condensed nuclei, which resulted in a significant decrease in cell viability. Earlier studies suggested a role for PBR ligands in the modulation of the apoptosis.[8,9,52–54] Likewise, PBR ligands increase apoptotic effect of some antitumoral drugs.[55,56] Hence, it has been suggested that PBR ligands could have applications in the treatment of cancer. Our results support the hypothesis that PBR ligands by themselves are insufficient to cause apoptosis but rather potentiate the proapoptotic effect of other drugs. Another interesting point is that these ligands show a nanomolar affinity for the PBR[57] but a concentration in the micromolar range is necessary to induce opening of the MPT pore.[58,59] Furthermore, the inducing or modulatory activity of PBR ligands on apoptosis have consistently demonstrated to occur within a micromolar range in tumour cell lines.[5,60,61]

Studies performed in tumour cells suggest several possible mechanisms involved in the proapoptotic properties of PBR ligands. Thus, it has recently been proposed that PBR ligands induce apoptosis in cancer cells through the activation of the p38/MAPK signalling pathway.[51] The activation of this pathway could also explain the antiproliferative properties of these drugs. Furthermore, other possible targets involved in the proapoptotic properties of PBR ligands are free radical production, and modulation of proteins acting on the mitochondrial control of apoptosis.[29,59,62,63]

Our results have demonstrated that colchicine does not increase the expression of PBR, as shown by RT-PCR and Western blot analysis indicating that the effect of PBR ligands is mainly due to an increase in its functionality, favouring MPT pore opening. As the PBR is believed to be a component of the MPT pore[1,3,64] we hypothesised that the mechanism through which PBR ligands potentiate colchicine-induced neurotoxicity depends on their role in

Figure 8. Cyt c is released during the apoptotic death of cultured cerebellar granule neurons. Co-localization with mitochondria assessed using Mito Tracker Red. Staining was either punctate in control cells, indicating that Cyt c is localized within the mitochondria, or not present, indicating that the cells had released it. Insert scale bar, 5 μm.



MPT functionality and its participation in the intrinsic pathway of apoptosis.

The pro-apoptotic effect of PBR ligands on colchicine-induced cell death led us to analyze caspase-3 activity. As we demonstrated previously, this enzyme was strongly activated by colchicine,[41,65] but the addition of PBR ligands did not induce a significant increase in this protease activity. However, the strength of caspase-3 activity makes it difficult to detect a potentiation by these compounds. Our results showed that colchicine-induced α-spectrin breakdown is significantly increased in the presence of PBR ligands, thereby implicating the caspase pathway. Consequently, we decided to study signals upstream to caspase activation in the intrinsic apoptotic pathway.

Since mitochondria regulate the apoptotic process, we studied the effects of PBR ligands on the release of proapoptotic proteins. The opening of the MPT pore liberates apoptogenic proteins, such as Cyt c and AIF from mitochondria to the cytosol and induces apoptosis via caspase-dependent and -independent mechanisms.[30,31,52,66] Our results indicated an amplification of Cyt c release that is translated into an increase in caspase-3 activity. This interpretation was confirmed by the observation that z-VAD-fmk, a pan-caspase inhibitor, blocked the apoptosis induced by colchicine and colchicine plus PBR ligands. AIF activity is affected by the tripeptide inhibitor z-VAD-fmk, which results in its partial inhibition, as described elsewhere.[67,68]

Nevertheless, our data also suggest the presence of an additional, caspase dependent apoptotic pathway, because we observed an increase in AIF release and translocation to the nucleus in colchicine-treated-cells that was markedly higher at 6 and 24 h in the presence of PBR ligands, again demonstrating increases opening of MPT. This observation could be explained by data indicating that caspase inhibition prevents the mitochondrial release of AIF, resulting in an antiapoptotic effect.[69]

In conclusion, our data demonstrate that PBR is present in CGCs and that PBR ligands enhanced colchicine-induced neurotoxicity. To our knowledge, this is the first study performed in neurons into a functional role of PBR ligands in apoptosis. We suggest that this proapoptotic effect may occur via facilitation of the intrinsic mitochondrial pathway, principally through the release of Cyt c and AIF.

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