

Anti-apoptotic effect of retinoic acid on retinal progenitor cells mediated by a protein kinase A-dependent mechanism

Roman Kholodenko¹, Irina Kholodenko¹, Viktor Sorokin², Anna Tolmazova¹, Olga Sazonova¹, Anton Buzdin¹

¹*Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry, 16/10 Miklukho-Maklaya, Moscow 117997, Russia;* ²*Russian State Medical University, Ostrovityanova 1, Moscow 117312, Russia*

Retinal progenitor cells (RPCs) are neural stem cells able to differentiate into any normal adult retinal cell type, except for pigment epithelial cells. Retinoic acid (RA) is a powerful growth/differentiation factor that generally causes growth inhibition, differentiation and/or apoptosis. In this study, we demonstrate that RA not only affects mouse RPC differentiation but also improves cell survival by reducing spontaneous apoptotic rate without affecting RPC proliferation. The enhanced cell survival was accompanied by a significant upregulation of the expression of protein kinase A (PKA) and several protein kinase C (PKC) isoforms. Treatment of cells grown in RA-free media with 8-bromoadenosine 3',5'-cyclic monophosphate, a known activator of PKA, resulted in an anti-apoptotic effect similar to that caused by RA; whereas the PKA inhibitor N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide dihydrochloride led to a significant (~32%) increase in apoptosis. In contrast, treatment of RPCs with any of two PKC selective inhibitors, 2,2',3,3',4,4'-hexahydroxy-1,1'-biphenyl-6,6'-dimethanol dimethyl ether and bisindolylmaleimide XI, led to diminished apoptosis; while a PKC activator, phorbol 12-myristate 13-acetate, increased apoptosis. These and other data suggest that the effect of RA on RPC survival is mostly due to the increased anti-apoptotic activity elicited by PKA, which might in turn be antagonized by PKC. Such a mechanism is a new example of tight regulation of important biological processes triggered by RA. Although the detailed mechanisms remain to be elucidated, we provide evidence that the pro-survival effect of RA on RPCs is not mediated by changed expression of p53 or bcl-2, and appears to be independent of β -amyloid, Fas ligand, TNF- α , ganglioside GM1 and ceramide C16-induced apoptotic pathways.

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Introduction

In mammals, retinogenesis mostly proceeds during embryo development and is completed within a few days after

birth. Retinal stem cells have the potential to produce either neural retinal progenitor cells (RPCs) or retinal pigment epithelial progenitors. RPCs generate seven known retinal neuronal cell types: rod and cone photoreceptor cells, required for black-white and color visions, respectively; Müller glia cells; bipolar, ganglion, horizontal and amacrine neurons, which perceive signals from photoreceptor cells, transform them and transduce them to the brain [1]. Although neuronal stem cell niches were found in brains of several mammalian species [2, 3], adult organisms are thought to lack any kind of neuronal retinal cell population renewal [4, 5]. Therefore, the treatment of various retinal disorders would require the use of neuronal stem cells to replace damaged retinal neurons or glia [6]. Indeed, in

Correspondence: Anton Buzdin

Tel: +7-495-330-6574; Fax: +7-495-330-6538

E-mail: anton@humgen.sibbc.ras.ru

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Abbreviations: retinal progenitor cells (RPC); all-trans-retinoic acid (RA); protein kinase C (PKC); protein kinase A (PKA); N-(2-[p-bromocinnamylamino]ethyl)-5-isoquinolinesulfonamide dihydrochloride (H89); 8-bromoadenosine 3',5'-cyclic monophosphate (8-Br-cAMP); 2,2',3,3',4,4'-hexahydroxy-1,1'-biphenyl-6,6'-dimethanol dimethyl ether (HBDDE); bisindolylmaleimide XI (BIM); phorbol 12-myristate 13-acetate (PMA)

animal models the approach of stem cell therapy was rather efficient for the treatment of diverse neurodegenerative diseases, such as Huntington's and Parkinson's diseases [7-9], as well as for curing some retinal disorders [10-12]. RPCs would be useful for retinal disease treatment because of their natural ability to differentiate into any of the above-mentioned retinal neuronal cell classes. Several inheritable and acquired retinopathies, including Retinitis Pigmentosa, are associated with the loss of particular retinal cell types [13].

It was shown that RPCs can be isolated from pre-natal or few days post-natal mouse [14], rat [15] and human [16, 17] retinas and can be cultured *in vitro*. Although there is a great difference between *in vivo* and *in vitro* stem cell differentiation and survival [18], the knowledge obtained from *in vitro* experiments will be necessary for the development of new *in vivo* assays and new therapeutic strategies. Ezeonu *et al.* [19] demonstrated that cell commitment in human retinal precursor cells could be modified in response to exogenous growth factors such as basic fibroblast growth factor and transforming growth factor- α . Moreover, RPC differentiation was density dependent: a larger proportion of multipotential precursors plated at a density of 1×10^4 cells/cm² differentiated into cells displaying photoreceptor phenotypes as compared with cells plated at higher densities, and the effects were enhanced in response to growth factors. Kelley *et al.* [20] used two photoreceptor-specific antibody markers, anti-recoverin (expressed in all photoreceptors) and anti-rhodopsin (specific for rod photoreceptors) to assess the effects of retinoic acid (RA) on differentiation of photoreceptor phenotypes *in vitro*. They found that RA treatment led to an increase in the number of cells expressing these markers after 2-8 days. This effect of RA on photoreceptor differentiation was specific, as the other major cell type produced at this stage in development, the amacrine cells, did not increase with RA treatment, but instead their productions were inhibited [21]. Recently, we have established a primary mouse RPC cell culture system and investigated the effects of various growth/differentiation factors on RPC phenotype and differentiation, including transforming growth factor- α , basic fibroblast growth factor, nerve growth factor, epidermal growth factor, platelet-derived growth factor, neurotrophin 3 and RA (unpublished data). The only factor found to reproducibly alter RPC phenotype was RA, while the others did not display any significant effects on RPC survival, morphology and marker protein expression, which might be due to the presence of these factors in the fetal serum used in culture media. RA-treated RPCs showed improved viability and decreased immunoreactivity to antibodies against nestin, a neuroepithelial stem cell marker

protein, whereas expression of the proliferative marker Ki 67 remained unchanged.

RA, known to be a powerful regulator of cell fate, regulates many aspects of development. In the developing retina, RA is produced at high concentrations, and several lines of evidence implicate a role for this molecule in photoreceptor development [21]. However, our observation that RA treatment leads to improved RPC viability represents a new finding, and the present study is focused on examining the underlying mechanisms. We report here that the effect of RA is due exclusively to a decrease in the spontaneous apoptotic rate of RPCs, through a mechanism most probably mediated by increase expression of protein kinase A (PKA), whose anti-apoptotic activity is in turn negatively modulated by protein kinase C (PKC).

Materials and Methods

Animals

All the experiments with mice were performed in accordance with both the Russian and the European Communities Council Directive (86/609/EEC) and institutional guidelines, and followed the tenets of the Declaration of Helsinki. In all cases, efforts were made to minimize suffering of the animals. CBA strain mice were used throughout the study. The plug date of timed pregnant mice was defined as embryonic day 0.

RPC isolation

Embryonic retinal tissues were isolated from day 14 mouse embryos. Eyes from 15 embryos were isolated and washed twice in $1 \times$ PBS standard buffer solution (ICN, USA) with added claforan (10 μ g/ml) and lincomycin (50 μ g/ml) antibiotics. Neural retinas were isolated by removing the retinal pigment epithelium and vitreous body. The retinas were then dissected and approximately 1 mm² fragments were incubated in 0.25% trypsin solution (PanEko, Russia) in PBS buffer for 30 min at 37 °C to homogenize the tissue. The trypsinization was then stopped by adding fetal calf serum (FCS) (HyClone, USA) to a concentration of 10%. The cells were washed twice in PBS, transferred to DMEM/F12 medium (Sigma, USA) supplemented with 10% FCS, and plated in six-well polystyrene culture plates (Corning Costar, USA) at a density of 5×10^5 cells/cm². Approximately 90% of the cells were viable. For viable cell counts, an aliquot was counted by the trypan blue (Sigma, USA) exclusion assay in a hemocytometer.

Media and culturing conditions

Media for cell culturing were DMEM/F12 (Sigma, USA) (0.2% NaHCO₃, pH 7.4) with 10% FCS, 2 mM L-glutamine (ICN, USA) and 25 μ g/ml linkomycin. Cultures were maintained at 37 °C in an incubator with 96% air and 4% CO₂. Cells were cultivated in multi-well plates or in cell culture flasks at densities of 5×10^5 , 1×10^5 , 5×10^4 and 1×10^4 cells/cm². Cell passaging was performed every 2-6 days, depending on the cultured cell proliferation rate, at 75% confluence. Cells were harvested by double washing with trypsin/versen solution (0.25% trypsin, 0.02% EDTA) in PBS and then centrifuged at $300 \times g$.

Monitoring of RA effects

To explore the effects of RA on cell survival and differentiation, RPCs of passage 8 were plated with different density on plastic culture dishes. For cell culture assays, DMSO-solubilized all-*trans*-RA (Sigma, USA) was added to cultured RPCs to a final concentration of 100 ng/ml. The culture medium was changed and the growth factors replenished every 2 days. The cells were monitored by dark-field and phase-contrast microscopy over a period of 1–7 days, and finally (after 7-day incubation) analyzed in immunocytochemistry experiments.

Light microscopy

Cell cultures were examined using an inverted light microscope Axio Vert 200 (Carl Zeiss, Germany). Samples were photographed with a digital camera AxioCam HRm (Carl Zeiss, Germany) at 10–80× magnification. For imaging and data analysis, the KS400 (version 3.0) software was used.

Immunocytochemistry

Cells for immunocytochemical and flow cytometry analyses were harvested with trypsin/versen solution (0.25% trypsin), double washed with PBS and fixed for 30 min with phosphate-buffered 2% paraformaldehyde (PanEko, Russia) at 4 °C. The fixed samples were washed twice in PBS and permeabilized in 0.6% saponin (Sigma, USA) PBS solution for 30 min at room temperature. The samples were then double washed with PBS containing 1% FCS and incubated for 1 h at 4 °C with the following primary anti-mouse antibodies (Santa Cruz, USA): anti-rhodopsin, mixed anti-green/blue opsins, anti-*nestin*, anti- β -arrestin, anti-calbindin, anti-calretinin, anti-recoverin, anti-Ki-67, anti-glial fibrillary acidic protein (GFAP) and anti-PCK α . For RA anti-apoptotic activity studies, anti-bcl-2 and anti-p53 antibodies (Sigma, USA) were used. Each representative sample of 5×10^5 cells was incubated with 2 μ g of primary antibodies in 100 μ l of 1×PBS/10% FCS. The samples were washed twice with 1×PBS/1% FCS, incubated for 1 h with fluorescein isothiocyanate (FITC)-conjugated secondary antibodies (Santa Cruz, USA) at 4 °C, and then washed and fixed in 2% paraformaldehyde solution, as described above. Immunoreactivity analyses with a minimum of 5 000 events per sample were performed on an EPICS Coulter XL-MCL flow cytometer. For fluorescent microscopy, cells were grown on glass slides in eight-well culturing plates. Such a visualization was used here as a raw control for flow cytometry results.

Staining with antibodies was performed as described above except that phycoerythrin-conjugated secondary antibodies (Santa Cruz, USA) were used. Nuclear stains were performed using 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI; Molecular Probes, USA). After antibody staining, cell nuclei were labeled with DAPI (0.1 μ g/ml, 5-min incubation) and washed twice with PBS. Samples were photographed with a digital camera AxioCam HRm (Carl Zeiss, Germany) at 10–80× magnification. For imaging and data analysis, the KS400 (version 3.0) software was used.

Propidium iodide staining for apoptotic cells and cell cycle analyses

Cell cycle/apoptosis analyses were performed using propidium iodide (PI) staining with subsequent FACS analysis. Prior to PI staining, cells were fixed in 70% ethanol (1 h incubation at 4°C), and double washed by suspending in PBS with a subsequent 10-min centrifugation at $300 \times g$. The cell pellets were further resuspended

in DNA staining solution (1×PBS, 20 μ g/ml propidium iodide (Merck), 50 μ g/ml RNase A (Fermentas)). For all samples, cell death analysis was performed in triplicate. An EPICS Coulter XL-MCL flow cytometer was used to evaluate apoptotic cell numbers with a minimum of 2 000 events analyzed per each sample. The data files were saved automatically by the CellQuest software (BD Biosciences), and WinMDI (version 2.8; The Scripps Institute, USA) was used to perform quadrant analysis. A two-tailed *t*-test to determine the significant difference between treatment and control experiments was performed using Excel (Microsoft). For cell cycle analyses, WinMDI and FCS Express programs were used.

Annexin V assay for apoptotic cell measurements

To confirm the results of PI staining for apoptotic cells, Annexin V assay kit (Molecular Probes) was used. RPCs were assayed by the detection of membrane externalization of phosphatidylserine with Annexin V-FITC conjugate. Briefly, 3×10^5 cells were harvested after 7-day RA treatment, washed twice with cold PBS and resuspended in 200 μ l of binding buffer. Both adherent and floating cells were harvested for the apoptosis assay. Annexin V-FITC and PI (1 μ g/ml) were added to individual samples and incubated for 15 min in a dark environment. The reaction was stopped by adding 300 μ l of binding buffer. Then the cells were analyzed with EPICS Coulter XL-MCL flow cytometer.

Cell growth/proliferation

For cell growth and proliferation studies, both colorimetric MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) and flow cytometric BrdU (bromo-2'-deoxyuridine) assays were performed. All MTT experiments were reproduced four times. An aliquot of the MTT solution (Sigma) was added to each sample for 4 h. The optical density (OD) was read in a Multiscan microplate reader (Titertek, Switzerland) at a test wavelength of 540 nm. Cell proliferation assay was based on BrdU incorporation into newly synthesized DNA of proliferating cells. Cells were plated in defined media supplied with 10% FCS containing 0–1 000 ng/ml RA for 7 days. During the last hour of incubation, BrdU was added to the cultures at 10 μ M. The solution was then removed and the cells were stained with FITC-coated anti BrdU antibodies according to a standard protocol [22].

Inhibitor/activator assay

The following protein kinase effector molecules were used: PKA inhibitor H89 (*N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide dihydrochloride), 10 μ M (Sigma); PKA activator 8-Br-cAMP (8-bromo-adenosine 3',5'-cyclic monophosphate), 10 μ M (Sigma); PKC inhibitors HBDDE (2,2',3,3',4,4'-hexahydroxy-1,1'-biphenyl-6,6'-dimethanol dimethyl ether), 5–10 μ M (Calbiochem), and BIM (bisindolylmaleimide XI), 1 μ M (Sigma); PKC activator PMA (phorbol 12-myristate 13-acetate), 10 μ M (Sigma). They were added separately or in combination to RPC cultures for a period of 7 days, and apoptotic cells were then analyzed by PI staining (see above).

Induction of apoptosis

RPCs were incubated for 3 days with the following known inducers of apoptosis in neuronal cells: β -amyloid (50 μ M), ceramide C16 (20 μ M), anti-Fas antibodies (1 μ g/ml), TNF- α (1 μ g/ml) and GM1 ganglioside (40 μ g/ml).

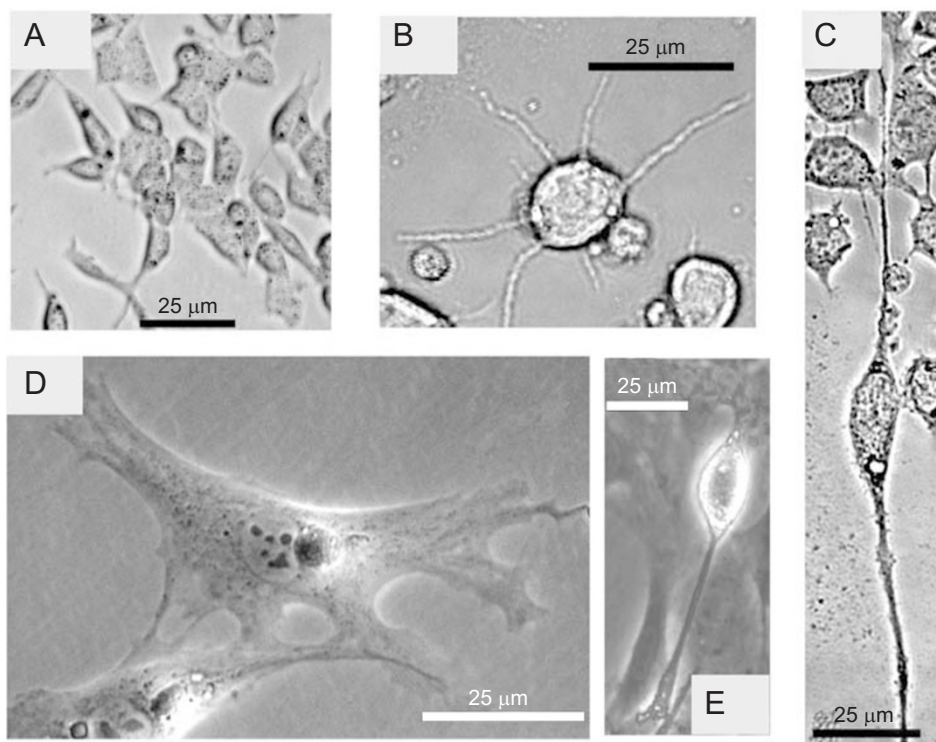


Figure 1 Phase-contrast photographs showing the morphologies of differentiated and undifferentiated RPCs. Magnification bar: 25 μ m. **(A)** Undifferentiated RPCs, 7 days after plating at a cell density of 5×10^5 cells/cm². **(B)** Differentiated RPCs displaying ganglion cell-like morphology. **(C)** Differentiated RPCs displaying bipolar neuron-like morphology. **(D)** Differentiated RPCs similar to astrocyte and Müller glia cells. **(E)** Photoreceptor-like morphologies of the differentiated RPCs.

DNA sequence analysis

Mouse genomic sequences and sequences of mouse gene transcripts were accessed through the UCSC genome browser (<http://genome.ucsc.edu/cgi-bin/hgBLAT>).

Oligonucleotides

Primers were synthesized using an ASM-102U DNA synthesizer (Biosan, Novosibirsk, Russia). The following oligonucleotides were used: PKC_A_for, 5'-gga ctt atc cac caa ggg atg-3' and PKC_A_rev, 5'-cca ggt tgt tgg atg gtt g-3' (for PKC α isoform expression); PKC_E_for, 5'-ctg gtc gct gcg cca tgc-3' and PKC_E_rev, 5'-atg ttg acg ctg aac cgt tg-3' (for PKC ϵ isoform expression); PKC_Z_for, 5'-tac agg tgc atc aac tgc aag-3' and PKC_Z_rev, 5'-cgt cgg cat caa gga gga-3' (for PKC ζ isoform expression); PKC_Q_for, 5'-gaa aca acc gtg gaa ctc tac tc-3' and PKC_Q_rev, 5'-agt cct tgc ctc gcc agc-3' (for PKC θ isoform expression); PKC_B_for, 5'-aag acc atc aag tgt tcc ctc a-3' and PKC_B_rev, 5'-tgc act cca cat cgt cat ctt-3' (for PKC β -1 isoform expression); PKC_G_for, 5'-cga ttt aat gta cca cat cca gc-3' and PKC_G_rev, 5'-acc tct ccc aat ega tcc aac-3' (for PKC γ isoform expression); PKC_D_for, 5'-gcc tca aca age aag gct aca-3' and PKC_D_rev, 5'-cag tcc gca gat gat ctc agc-3' (for PKC δ isoform expression); PKC_I_for, 5'-gat ccg cat ccc gcg ttc-3' and PKC_I_rev, 5'-cac tct cga atc ctg cct ctg a-3' (for PKC λ/ι isoform expression); PKC_H_for, 5'-gaa gac gag tcc atc aag tga ac-3' and PKC_H_rev, 5'-gcc gaa tct gct gga aga g-3' (for PKC β

isoform expression); PKA_A_for, 5'-tcc tgg ggg aca ccc tgg gcg-3' and PKA_A_rev, 5'-ggc ttc gtt gtg ttg agt gca tcc-3' (for PKA α catalytic subunit expression); PKA_B_for, 5'-caa gag gct tgc aaa gac atc-3' and PKA_B_rev, 5'-ccg ttc tct tca act tct gat tta c-3' (for PKA β regulatory subunit expression).

RNA isolation and cDNA synthesis

Total RNA was isolated from frozen cells pulverized in liquid nitrogen using an RNeasy Mini RNA purification kit (Qiagen). All RNA samples were further treated with DNase I to remove residual DNA. Full-length cDNA samples were obtained according to a cap switch effect-based SMART cDNA synthesis protocol (Clontech, BD Biosciences) using an oligo (dT)-containing primer and PowerScript reverse transcriptase (Clontech, BD Biosciences).

RT-PCR

All RT-PCR experiments described in this section were reproduced at least three times using independent cDNA preparations. For RT-PCR control of cDNA quality, we used two primer sets specific for the β -actin gene transcript 5' and 3' parts, respectively (Gene Checker Kit, Invitrogen). To find out transcription levels of selected genes in RPC samples under study, we performed a series of RT-PCR experiments with primers predominantly designed against neighboring constitutive exons in the middle part of the corresponding cDNA molecule. Prior to the RT-PCR analysis, the priming efficiency of the

primers was pre-examined by genomic PCRs at temperatures varying depending on the primer combination used. These PCRs were carried out for 19, 22, 25 and 28 cycles, with 40 ng of the mouse genomic DNA isolated from RPCs using a Wizard SV Genomic DNA Purification System (Promega). The RT-PCR was performed with cDNA samples obtained from RA-treated and untreated RPCs, an equivalent of 20 ng total RNA being used as template in each PCR reaction performed in a final volume of 40 μ l. Aliquots (5 μ l) of the reaction mixture after 21, 24, 27, 30, 33, 36 and 39 cycles of the amplification were analyzed by electrophoresis in 1.5% agarose gels. In all cases, the transcriptional status was determined from the number of PCR cycles needed to detect a PCR product of the expected length, and the PCR product concentration was measured using a Phomat system and the Gel Pro Analyzer software.

Results

Cultured RPCs display characteristic morphologies and express marker proteins of retinal neurons

Multipotential RPCs were isolated from day 14 mouse embryos, as the differentiation of retinal precursors has not yet been completed at this time point [1]. To characterize the primary cell culture, we investigated its ability to display typical retinal cell phenotypes, either by expressing marker proteins or by demonstrating characteristic cell shapes. In line with previous findings [19], the major RPC differentiation factor was the cell density: more than 70% of the cells plated at the densities of 1×10^4 or $5 \times 10^4/\text{cm}^2$ differentiated into various retinal neuron-like cell types, in contrast to cells plated at high density which tended to retain their undifferentiated phenotype (Figure 1A). Another fac-

tor was the concentration of FCS in the media, which varied from 2% to 10%. Low serum content resulted in poor cell survival after 4 days of cultivation, whereas 5% and 10% FCS were optimal for cell proliferation and differentiation. For both the 1×10^4 and $5 \times 10^4/\text{cm}^2$ densities, the observed cellular phenotypes were as follows: retinal ganglion cell-like phenotype (4-6% of the cells, Figure 1B), bipolar cell-like phenotype (5-10%; Figure 1C), astrocyte and Müller glia-like cells (5-8%; Figure 1D), retinal photoreceptor-like cells (3-6%; Figure 1E), as well as other cells with neuronal morphology, which could be considered ganglion, bipolar, horizontal and amacrine-like cells (40-50% of the cells), and undifferentiated cells (20-27%).

In further experiments, RPCs plated at the medium density (5×10^4 cells/ cm^2) were used throughout the study. Cultured RPCs were antibody-stained for retinal marker proteins: blue and green opsins, expressed in cones; rod marker rhodopsin; β -arrestin and recoverin from both photoreceptor cell types; calbindin, PKC α isoform and calretinin, which are markers for horizontal cells, bipolar cells and retinal amacrine interneurons, respectively; GFAP, characteristic of Müller glia cells, as well as for the proliferating cell marker Ki 67 and neuroepithelial stem cell marker nestin. Cell staining was analyzed both by flow cytometry (Table 1) and fluorescent microscopy. Cells were found to be immunoreactive against all of the above marker protein-specific antibodies at very different levels ranging from 3.6% of the cells for GFAP to 43.1% for green/blue opsins. These data strongly support the RPC

Table 1 Flow cytometry data on marker protein expression in RPCs

Marker protein ^a	Passage 10 ^b	Passage 10+RA ^c	% of expressed cells	
			Passage 50 ^b	Passage 50+RA ^c
GFAP	3.6 \pm 1.1	4.9 \pm 0.9	2.6 \pm 0.7	4.2 \pm 0.9
Calbindin	33.6 \pm 2.7	36.64 \pm 2.8	34.5 \pm 2.5	18.4 \pm 1.4
Calretinin	26.1 \pm 1.9	22.8 \pm 1.9	23.6 \pm 2.0	21.6 \pm 1.9
β -Arrestin	27.6 \pm 2.1	14.3 \pm 1.5	16.8 \pm 1.7	12.3 \pm 1.2
Recoverin	36.7 \pm 2.1	25.6 \pm 1.8	34.8 \pm 2.2	14.2 \pm 0.9
Protein kinase C isoform α	4.4 \pm 1.1	11.0 \pm 1.4	5.2 \pm 1.0	17.3 \pm 1.3
Rhodopsin	12.1 \pm 0.9	23.6 \pm 1.1	6.8 \pm 0.9	13.1 \pm 1.4
Green/ blue opsins	30.4 \pm 2.7	43.1 \pm 2.1	21.8 \pm 2.3	35.7 \pm 2.2
Nestin	8.1 \pm 1.2	5.5 \pm 0.9	12.2 \pm 1.8	7.3 \pm 1.0
Ki 67	30.3 \pm 3.0	26.8 \pm 2.1	55.1 \pm 2.0	28.5 \pm 1.3
Bcl-2	1.2 \pm 0.3	1.3 \pm 0.5	ND	ND
P53	6.1 \pm 0.7	7.3 \pm 0.9	ND	ND

^aMarker protein expression investigated in at least three independent experiments.

^bWithout RA.

^cWith RA added to the medium.

origin of the isolated cell culture. During at least first 15 passages, the cell culture entirely retained all of the above-mentioned morphological and marker protein expression patterns, as examined visually and immunocytochemically for every fifth passage of the RPC culturing. Like Ezeonu *et al.* [19], we have been able to passage these cells up to the 50th passage. However, by passage 20 RPCs have lost the ability to differentiate into cells with the morphology of mature retinal neuronal cells. Also, these cells did not keep their marker protein expression patterns. Therefore, multipotent RPCs from passage 10 were used for further experiments.

RA affects RPC viability by reducing apoptosis

We attempted to study the influence of RA on the RPC phenotype. RA was added to 5% FCS culture media, and the resulting cell phenotypes were analyzed 7 days later both visually and by flow cytometry. In good agreement with previous data, RA-treated RPCs demonstrated an altered expression of several marker proteins [21] (Table 1). However, RA treatment did not alter RPC morphologies, and the relative ratios of photoreceptor, amacrine, bipolar, ganglion and Müller glia cell-like morphotypes remained essentially the same. Unexpectedly, RA-treated cells were characterized by an improved viability as the treatment led to 1.84 ± 0.1 -fold higher cell yield after 7 days of culturing in a series of six independent experiments whereby viable cells were examined by the trypan blue exclusion assay. Interestingly, essentially the same RA effect (increased cell viability and altered characteristic marker protein expression profile) was observed for RPCs in passage 50 as well, when the cells were unable to develop adult retinal neuron-like shapes. These data suggest that RA affects both RPC survival and phenotype.

In order to investigate whether the enhanced RPC survival is due to reduced apoptosis or a proliferative activity of RA, we performed several assays. For cell growth and proliferation studies, both colorimetric MTT and flow cytometric BrdU assays were performed. A PI analysis revealed that RPC cell cycle progression was not affected by RA treatment. Both MTT- and BrdU-based proliferation assays showed that RA had no effect on RPC proliferation as well (Figure 2). RA effects on RPC proliferation were monitored 24-h after the addition of RA using the MTT reagent and 7 days after by BrdU staining. The main message of Figure 2A is that RA has no initial toxic effect on RPCs in a wide range of concentrations. For a short period of incubation with RA like 24 h, no significant RA effect on RPC viability is visible. However, for a period of 7 days this effect was significant (1.7-1.9 greater cell yield when RA was added, see above), whereas the proportion of proliferating cells remained essentially the same (Figure 2B), thus suggesting

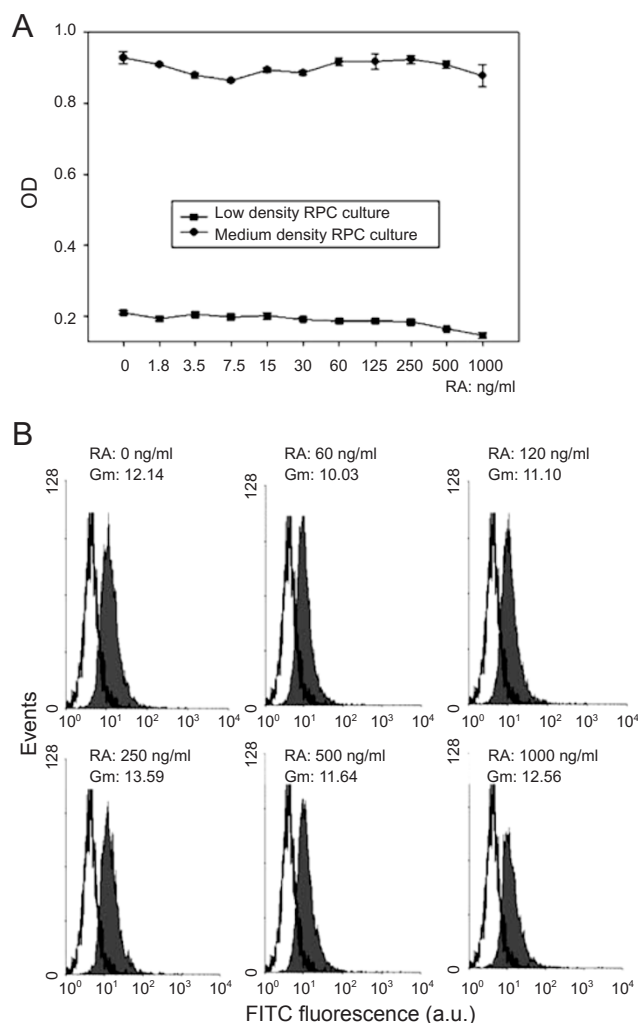


Figure 2 RA effect on RPC proliferation. **(A)** MTT assay. Low and medium density RPC cultures (1×10^4 and 5×10^4 cells/cm², respectively) exposed to different RA concentrations ranging from 0 to 1000 ng/ml were incubated with the MTT reagent. The cells were washed, solubilized with 10% SDS, and the OD was measured at 570 nm. **(B)** BrdU assay. Flow cytometry analyses of BrdU incorporation in RPCs exposed to different concentrations of RA, using FITC fluorescence (a.u.). Left peaks (transparent) – control fluorescence (BrdU-free culture medium); right peaks (dense) – 10 μ M BrdU added to the medium. RA concentrations and fluorescence peak positions (Gm) are indicated.

that the increased RPC viability mediated by RA is not due to an enhanced RPC proliferation. It should be mentioned here that the proliferative marker Ki 67 expression was observed at essentially the same level for both RA-treated and untreated RPCs from passage 10 ($26.8 \pm 2.1\%$ and $30.3 \pm 3.0\%$ cells, respectively) and for RA-treated RPCs from passage 50 ($28.5 \pm 1.3\%$ cells). In our experiments,

untreated RPCs from passage 50 demonstrated ~twofold higher expression of Ki 67 ($55.1 \pm 2.0\%$ cells) (Table 1). However, this increased expression of Ki 67 did not correlate with increased proliferation rate, as measured in BrdU staining experiments; instead, the proliferation rate was shown to be the same for both RA-treated and untreated RPCs from both passages 10 and 50. Although Ki 67 is a widely used marker, it is known that the data on the expression of different proliferative marker proteins, such as Ki 67, PCNA or Mcm-2, do not always accurately reflect cell proliferation status, and that other, more direct approaches like BrdU staining are preferred for counting proliferating cells. Moreover, the biological function of Ki 67 remains

unclear. Therefore, the enhanced Ki 67 expression by the passage 50 RPCs might be unrelated to cell division.

To monitor the effect of RA on apoptosis in RPCs, we used the PI-based screening system. The data (Table 2) demonstrated that for RPCs from passages 10 and 50, plated at both medium and high densities, addition of RA to the culture media led to ~23% decrease in the number of apoptotic cells. To confirm the PI screening results, we employed the Annexin V assay. Importantly, a similar decrease (by ~23%) in the number of apoptotic cells was observed for RA-treated cells from both passage 10 and passage 50 using this approach (Figure 3), thus demonstrating the reliability of the PI-based system for apoptosis

Table 2 Influence of different factors on apoptosis in RPCs

Factors	Apoptotic cells (%) ¹	Effect (%) ²	Comments ³
–	23.1±0.9	–	Controls
RA	17.7±0.9	–23.4	Retinoic acid added (+RA)
–	32.6±1.0	–	High cell density (5×10^5 cells/cm ²)
RA	25.2±1.2	–22.7	High cell density + RA
–	20.7±0.8	–	RPCs at passage 50
RA	15.7±0.8	–24.2	RPCs at passage 50 + RA
–	29.5±1.2	–	RPCs at passage 50, high cell density
RA	22.5±0.7	–23.7	RPCs at passage 50, high cell density + RA
β-Amyloid	44.5±1.4	+92.6	β-Amyloid
β-Amyloid + RA	42.2±1.7	+82.7	β-Amyloid + RA
C16-Cer	26.0±0.7	+12.6	Ceramide C16
C16-Cer + RA	25.7±1.0	+11.3	Ceramide C16 + RA
Anti-Fas	23.5±1.0	+1.7	Anti-Fas antibodies, Fas ligand functional analog
Anti-Fas + RA	23.9±0.7	+3.5	Anti-Fas antibodies+RA
GM1	26.2±0.6	+13.4	Ganglioside GM1
GM1 + RA	26.6±0.9	+15.2	Ganglioside GM1 + RA
TNF α	23.8±1.1	+3.0	Tumor necrosis factor α
TNF α + RA	22.9±1.0	–0.9	Tumor necrosis factor α + RA
H89	27.9±1.1	+20.9	PKA inhibitor
H89 + RA	30.5±0.9	+31.9	PKA inhibitor + RA
8-Br-cAMP	19.9±0.7	–13.9	PKA activator
8-Br-cAMP + RA	17.6±0.3	–23.9	PKA activator + RA
HBDDE	16.0±0.8	–30.5	PKC inhibitor
HBDDE + RA	12.6±0.4	–45.6	PKC inhibitor + RA
BIM	18.4±0.4	–20.5	PKC inhibitor
BIM + RA	16.2±0.9	–29.7	PKC inhibitor + RA
PMA	25.9±1.1	+12.0	PKC activator
PMA + RA	18.6±0.8	–19.3	PKC activator + RA

¹Percentage of apoptotic RPCs found with the PI assay from at least three independent experiments.

²Effect on RPC apoptosis, percentage relative to the corresponding controls.

³When not specified, RPCs from passage 10 plated at a density of 5×10^4 cells/cm².

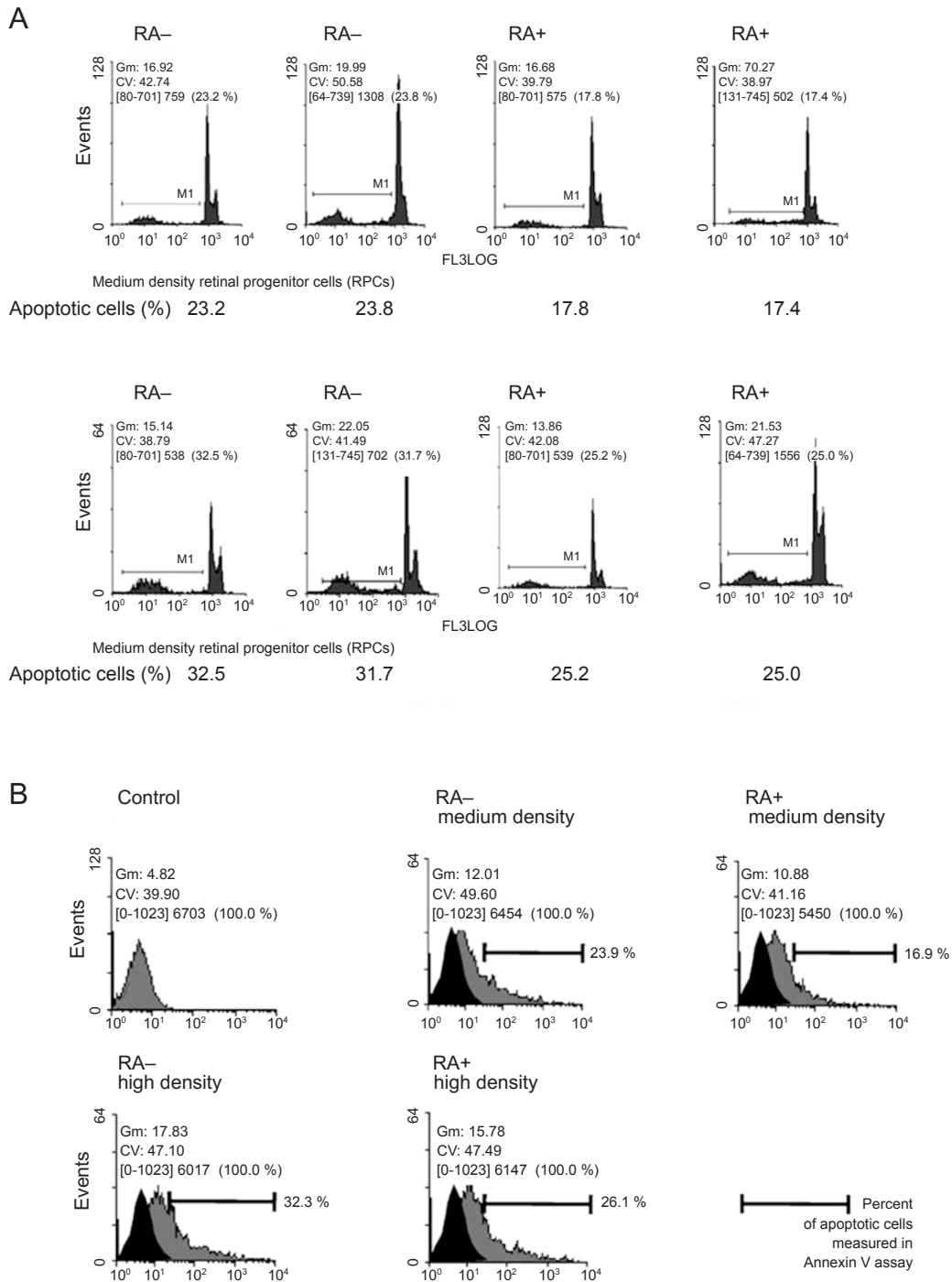


Figure 3 Long-term effect of RA on RPC apoptosis. RPCs were harvested after 7 days of RA treatment and analyzed with either PI or Annexin V staining assays. Both assays produce a consistent result of ~23% decrease in apoptotic cells when RA is added to the culture media for both medium- and high-density plated RPCs. **(A)** Representative results of the PI assay. Medium- and high-density RPC cultures (5×10^4 and 5×10^5 cells/cm²) were grown in RA-containing or in RA-free medium. Apoptotic cells were measured in a PI-based flow-cytometer assay. Each representative histogram is shown in duplicate. **(B)** Representative histograms of the Annexin V assay. Medium- and high-density RPCs were grown in RA-containing or in RA-free medium. Apoptotic cells were directly stained with Annexin V-FITC conjugate and measured on a flow cytometer. Control experiment: attached RPCs stained after 24-h culturing in RA-free medium with 10% FCS.

measuring in this study. This decrease of ~23% seems reasonable to explain the approximately twofold higher cell yield in RA-treated RPC culture after 7 days. Indeed, even ~10% decrease in apoptotic human HL-60 cells provided significantly higher augmentation of the cell yield [23]. We, therefore, conclude that the observed effect of RA on RPC viability is exclusively due to the RA-mediated anti-apoptotic activity in these cells.

PKA and PKC antagonistic activities are probable mediators for the RA-induced anti-apoptotic effect

We further tried to explore the mechanisms underlying the anti-apoptotic effect of RA in RPCs. Many apoptotic pathways known to be activated by specific proteins or chemicals operate in neuronal cells, although the specific details may differ depending on the tissue origin. RA interference with any of such pathways would provide a hint for understanding of the mechanism of its anti-apoptotic activity. To assess RA effects, the following known apoptosis inducers were used: β -amyloid, ceramide C16, anti-Fas antibodies (serve as *in vitro* functional analogs of Fas ligand), TNF- α and GM1 ganglioside. Of these, only β -amyloid, ceramide C16 and ganglioside GM1 were able to induce apoptosis in RPCs as manifested by a 92%, 13% and 13% increase in the apoptotic cell number, respectively, compared with controls. However, RA apparently did not interfere with any of the apoptotic inducers mentioned above (Table 2), thus suggesting that its effect might involve other signaling pathway(s). As bcl-2 and P53 proteins are among the most important players in signaling cascades responsible for regulating cell death and survival, we further antibody-stained RA-treated and untreated RPCs for these proteins. Somewhat unexpectedly, the expression of these markers was unaffected by the addition of RA to the culture medium (Table 1).

However, as revealed by a previous marker protein expression assay, expression of the PKC α isoform was upregulated in RA-treated cells (Table 1). PKC α is known to be not only a bipolar cell marker, but also one of the key Ser/Thr protein kinases involved in multiple signal-transduction pathways. RT-PCR analyses with primers specific to nine different PKC isoforms revealed that the transcription of four of them (PKC α , ϵ , ζ and λ/ι) was ~four- to eight-fold upregulated in RA-treated cells (Figure 4A), with the PKC ζ transcript level being the highest. The other PKC isoforms were expressed at similar levels in both treated and untreated cells. To address the question as to whether the effect of RA is due to an increased PKC activity, we performed assays with two known PKC inhibitors, HBDDE and BIM, and with the specific PKC activator PMA. Surprisingly, PKC activation resulted in increased apoptosis (+12% as compared with controls), whereas PKC inhibition with both

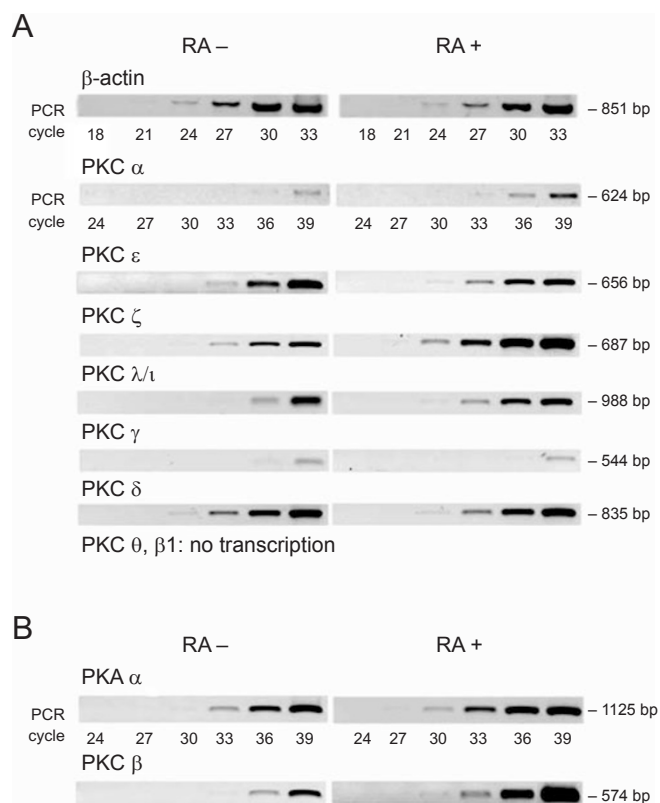


Figure 4 Representative results of RT-PCR analyses of cDNAs obtained from RA-treated (RA+) and untreated (RA-) RPCs. All RT-PCR experiments were reproduced at least three times using independent cDNA preparations. **(A)** RT-PCR amplification with primers specific to β -actin (control amplification serving as an internal standard) and to different PKC isoforms: PKC α , ϵ , ζ , λ/ι , γ , δ , θ and $\beta 1$. **(B)** RT-PCR amplification with primers specific to PKA catalytic α and regulatory β subunits. PCR product lengths are indicated.

HBDDE and BIM decreased the proportion of apoptotic cells (-31% and -21%, respectively; Table 2). Thus, RA-induced PKC activation cannot explain the anti-apoptotic effect of RA on RPCs. We hypothesized that the effect of RA might be mediated by another protein kinase, known as cAMP-dependent Ser/Thr protein kinase, or PKA. It is known from the literatures that in many cases both PKC and PKA are activated by RA [24]; more interestingly, they frequently act as functional antagonists of each other [25]. To test this hypothesis, we performed RT-PCR experiments with primers specific to two PKA subunits: catalytic α and regulatory β . RT-PCRs revealed that the expression of both PKA subunits was ~eight-fold upregulated in RA-treated cells, thus suggesting PKA activation by RA (Figure 4B). This hypothesis was further supported by experiments with the PKA inhibitor H89 and activator 8-Br-cAMP (Table

2). PKA activation resulted in reduced apoptosis (−14%), whereas PKA inhibition increased RPC apoptosis (+21%). Interestingly, when PKA activator and RA were added together to RPCs, the effect on RPC apoptosis was almost identical to that caused by RA alone, consistent with the notion that PKA acts in the RA-mediated pathway. We, therefore, conclude that RA stimulation of RPCs increases the expression of both PKA and PKC, which may act as functional antagonists by decreasing and increasing apoptosis, respectively. Such double kinase activation might be advantageous for tight regulation of this important process by providing an instrument for a quick and balanced response to new external or intracellular signals.

Discussion

Our study suggests RA-mediated activation of two protein kinases (PKA and PKC) acting as functional an-

tagonists. The PKA/PKC inhibitor-based analyses provide another line of evidence for such a hypothesis (Figure 5A). According to the model, RA stimulation activates both PKA and PKC, with the former supporting RPC survival, whereas the latter increasing apoptosis (Figure 5B). This model predicts that RA stimulation of RPCs accompanied by treatment with PKC inhibitors should result in a greater anti-apoptotic activity than that induced by RA alone. In this case PKA will be stimulated, while the PKC-mediated compensatory mechanism, normally suppressing the PKA effect, will be inhibited (Figure 5C). This prediction was tested by an inhibitor assay: treatment with RA and the PKC inhibitor indeed resulted in a significantly better anti-apoptotic effect (−46% and −30% for HBDDE and BIM, respectively) than that of RA alone (−23%). In contrast, PKA inhibition together with RA treatment would cause PKC activation that is not balanced by PKA (Figure 5D), and therefore would lead to increased apoptosis compared with cells treated with RA alone or untreated cells. This prediction was also confirmed by experimental data (+32% vs −23% and 0%, respectively).

It was previously reported that PKA and PKC may serve as antagonists for each other in RA-treated cells, which is in good agreement with the present findings. For instance, in Sertoli cells from rat testis RA induced differentiation, with the effect of RA being enhanced by PKC activation and reduced by upregulation of PKA [24]. In HepG2 cells, both RA treatment and PKA activation resulted in upregulation of the cholesterol seven α -hydroxylase gene transcription, whereas PKC activation led to silencing of this gene [25]. In human neuroblastoma, PKC activation mediated an anti-apoptotic effect via Bcl-2 activation, whereas PKA suppressed Bcl-2 activation and increased the proportion of apoptotic cells [26]. An overwhelming majority of RA-induced effects in neuronal cells, as described in the literature, have been ascribed to cell maturation, differentiation, control of proliferation or to cell death, with very few published examples of RA-mediated anti-apoptotic effects. For retinal cells, only indirect evidence was obtained recently: Zhou *et al.* [27] reported that inhibition of RA receptors in the developing mouse eye stimulated apoptosis in neonatal mice retinas. In this paper, we propose a mechanism for the anti-apoptotic activity of RA in RPCs, which comprises activation of PKA and PKC. Interestingly, structural data support the idea that several PKC isoforms, normally activated by increased concentrations of diacylglycerol and calcium ions, could also be directly activated by RA, as they contain potential binding sites for two RA molecules [28]. An interesting model for PKC and PKA double activation (although not RA-dependent) was previously suggested for B lymphocytes: high intracellular calcium concentrations via calmodulin lead to increased cAMP concentration,

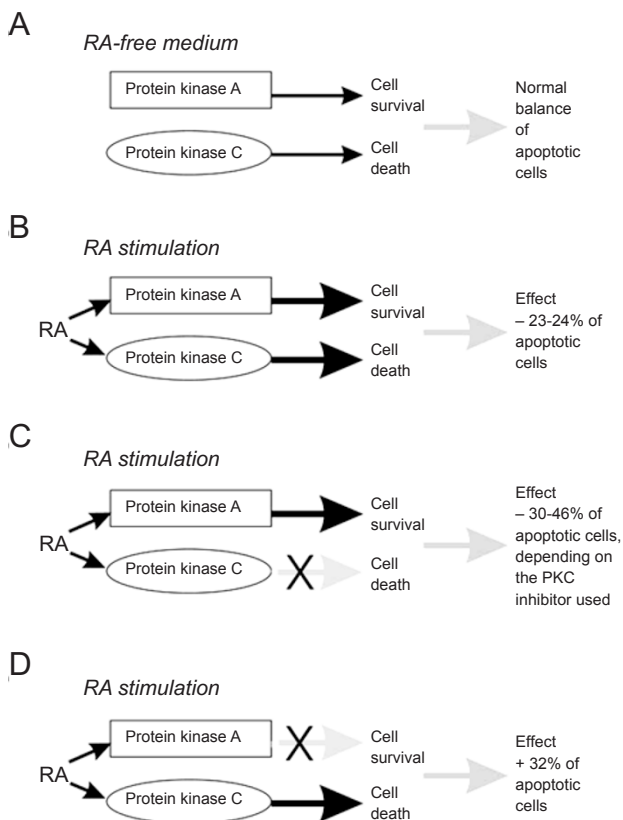


Figure 5 A model explaining the effects of RA and protein kinase inhibitors on RPC survival. Full data sets are presented in **Table 2**. (A) No RA or protein kinase inhibitors added to RPCs. (B) RA added to the culture medium. (C) RA and PKC inhibitors (BIM or HBDDE) added to the medium. (D) RA and PKA inhibitor (H89) added to the medium.

which modulates PKA activation. Simultaneously, calcium ions activate PKC isoforms α , δ and ϵ (in our experiments, transcription of α , ϵ , ζ and λ/ι isoforms was activated by RA). The mechanism proposed in our model (Figure 5) relies mostly on the inhibitor/activator studies performed with well-known PKA and PKC inhibitors and activators: H89 [29], 8-Br-cAMP [30], HBDDE [31], BIM [32] and PMA [33]. A more detailed analysis of RA-mediated anti-apoptotic mechanisms is a subject for future studies. The results reported here might be of considerable usefulness in creating better *in vitro* RPC systems with increased cell viability for modeling eye development and/or for future stem cell-based strategies to treat retinopathy.

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