PKC alpha affects cell cycle progression and proliferation in human RPE cells through the downregulation of p27kip1

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Purpose: Protein kinase C (PKC) plays an important role in the regulation of retinal pigment epithelium (RPE) cell proliferation. In this study, we investigated which of these isozymes could be responsible for the cell cycle and proliferation in human RPE cells.

Methods: The effect of PKC activators on human RPE cell cycle progression was tested by flow cytometry. To identify the isoform of PKC responsible for the increased progression of the cells through the cell cycle, we monitored the effect of phorbol 12-myristate 13-acetate (PMA) on the subcellular localization of the nine PKC isoforms expressed in RPE cells. To evaluate the molecular mechanism by which PKCα induces cell cycle progression, we examined the transcript, protein, and cellular levels of cell cycle regulatory proteins using RT–PCR, western blotting, and a confocal microscope, respectively.

Results: We demonstrated that PKC activation by PMA affected cell cycle progression in RPE cells. Of the nine PKC isoforms that were present in RPE cells, we found PKCα was both necessary and sufficient to promote cell cycle progression after being stimulated with PMA. Decreased PKCα expression resulted in a significant decrease in cell proliferation. The only cell cycle-regulatory molecule whose expression was rapidly altered and decreased by PKCα activity was the cyclin-dependent kinase (CDK) inhibitor p27kip1.

Conclusions: These results suggest that PKCα affects cell cycle progression and proliferation in human RPE cells through the downregulation of p27kip1.

Protein kinase C (PKC) is a multigene family of phospholipid-dependent serine-threonine kinases that mediates the phosphorylation of numerous protein substrates in signal transduction. It plays a central role in cellular processes such as proliferation, differentiation, mitosis, and inflammatory reactions [1,2]. Up to now, at least 12 isoforms of PKC have been cloned to date, all displaying different enzymatic properties, tissue expression, and intracellular localization [3,4]. PKCs are divided into three major groups according to the variability of their regulatory domains. The classic PKCs (cPKCs: PKCα, PKCβII, and PKCγ) require calcium, phosphatidylserine, and diacylglycerol (DAG), or phorbol esters, for full activation. The novel PKCs (nPKCs: PKCδ, PKCε, PKCζ, PKCη, and probably PKCλ [5]) do not require calcium or their activation. The third group are the atypical PKCs (aPKCs: PKCα, PKCδ, and PKCε), whose activation depends on phosphatidylserine, but not on DAG, nor on calcium or phorbol esters. The differences in function of specific PKC isoforms are mainly due to their subcellular localization, their activation or inhibition by different stimuli, and transcriptional regulation [6,7].

It has been well documented that the PKC family is involved in the processes of proliferation, migration, phagocytosis, and gel contraction in retinal pigment epithelium (RPE) cells [8-14], which have all been implicated in the pathogenesis of proliferative vitreoretinopathy (PVR). For example, Harris et al. reported that hypericin, a specific inhibitor of PKC, could have potential as a therapeutic drug for PVR and that its antiproliferative and apoptotic effects on RPE cells in vitro were in part mediated by PKCα [9]. Another study showed that the PKC inhibitor calphostin C dramatically affected the growth rate of RPE cells [10]. We have found that hypericin has potential as a therapeutic drug for PVR, potentially through its inhibition of the Ca2+ influx pathway [15]. Rabbit models have shown that intravitreal injection of hypericin is also a safe and effective means of reducing experimental PVR [16,17]. However, since the distribution of PKC isoforms is both tissue-specific and cell type-specific [18], the PKC activity is the sum of the isoforms expressed in that tissue. Therefore, data regarding the precise pattern of isoform expression in RPE cells could be informative with regard to their physiologic regulation and potential role in PVR [19]. Our previous study characterized the expression pattern of all 12 PKC isoforms and showed that 10 isoforms (PKCα, PKCβII, PKCδ, PKCε, PKCθ, PKCι, PKCλ, PKCν, PKCω, and PKCε) were present in cultured human RPE cells [20]. This identification provides the first step toward elucidating their roles in RPE cell proliferation. In this study, we further investigated which of these isoforms could be responsible for the cell cycle in human RPE cells. Our results demonstrate that PKCα controls proliferation and...
regulates cell cycle progression in RPE cells through the downregulation of cyclin-dependent kinase (CDK) inhibitor p27\(^\text{kip1}\).

**METHODS**

*Reagents:* Trizol reagent was obtained from Life Technologies (Gaithersburg, MD). The SuperScript™ first strand synthesis system was obtained from Invitrogen (Carlsbad, CA). The enhanced chemiluminescence (ECL) kit for western blotting was from Cell Signaling (Danvers, MA). Rabbit polyclonal antibodies against p27 and phorbol 12-myristate 13-acetate (PMA) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal PK\(_{\alpha}\), PK\(_{\gamma}\), PKC\(_{\delta}\), PKC\(_{\eta}\), PKC\(_{\zeta}\), and PKC\(_{\theta}\) antibodies were purchased from BD Systems (Torrance, CA). Monoclonal PKC\(_{\beta}\), PKC\(_{\beta2}\), PKC\(_{\varepsilon}\), and PKC\(_{\mu}\) antibodies were from Sigma (St. Louis, MO). Anti-β actin was purchased from Boster (Dickinson). 

Fluorescence intensities with Lysis II software (Becton Dickinson). The red fluorescence (PI) from cells were excited at 488 nm using channel 2. The distribution of cells in different phases of the cell cycle was obtained by analyzing fluorescence intensities with Lysis II software (Becton Dickinson).

*Preparation of cell extracts:* The medium was removed and washed twice with ice-cold PBS. The human RPE cells were lysed with sample buffer that contained 60 mM Tris, pH 6.8, 2% (w/v) SDS, 100 mM 2-mercaptoethanol, and 0.01% (w/v) bromophenol blue [21]. The lysates were then incubated on ice for 30 min. The extracts were harvested using a cell scraper, then boiled for 5 min and stored at –20 °C.

*Western blot analysis:* Cells extracts from confluent human RPE cells were processed for western blot analysis [22]. Briefly, 40 µg of protein per well was loaded on a 12% sodium dodecyl sulfate- PAGE (SDS–PAGE) gel. Protein was electrophoresed to polyvinylidene difluoride membranes (Millipore) for 2 h at 350 mA, then blocked with a solution of Tris-buffered saline (TBS) containing 5% nonfat milk and 0.1% Tween-20 (TBST) for 1 h, and incubated with primary antibodies overnight at 4 °C. After three washes with TBST for 10 min at room temperature, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody for 3 h at room temperature, and then washed one time with TBST for 30 min at room temperature. Localization of antibodies was detected by chemiluminescence using an ECL kit following the manufacturer’s instructions. Each PKC isoform was examined in a minimum of four independent experiments. As recommended by the supplier of the primary antibodies, we used mouse brain lysate as a positive control.

*Subcellular fractionation:* Confluent cells were partitioned into soluble and particulate fractions, using a method previously described [23,24]. Briefly, cells were lysed in digitonin lysis buffer (as described in the previous section, but without Triton X-100) and homogenized for 10 s at 3300× g Digitonin-soluble (cytosolic) and insoluble (particulate) fractions were separated by ultracentrifugation at 100,000× g for 45 min at 4 °C. Supernatant was collected, and it formed the cytosolic fraction. The pellet was resuspended in digitonin buffer containing 1% Triton X-100, incubated on ice for 30 min, and cleared by centrifugation for 10 min at 10,000× g at 4 °C. Proteins were quantified by the Bio-Rad protein assay. Samples were subjected to SDS–PAGE as described in the previous section; 80 µg of protein were loaded per well.

*Cell proliferation by thymeleatoxin or siRNA-PKCα:* RPE cells were cultured in 75 mm dishes (10×10⁵ cells/dish) in

**Reverse transcription-polymerase chain reaction:** Total RNA was extracted using Trizol reagent according to the manufacturer’s procedure. The integrity of the RNA was checked by 2% agarose gel electrophoresis. Approximately 5 µg RNA was reverse-transcribed following the protocol of the SuperScript™ first-strand synthesis system. cDNAs encoding the cell cycle regulator genes were amplified by PCR as follows: denaturation for 30 s, annealing for 30 s and elongation at 72 °C for 60 s. Primer sequences were designed using Primer 3, as shown in Table 1. Each PCR was done a minimum of three times with each set of primers. PCR products were analyzed by agarose (2%) gel electrophoresis and ethidium bromide staining.

*Human RPE cell culture:* Human RPE cells were isolated from five human donors, age 23 to 40 years, within 24 h after death, which were obtained from the Zhongshan Ophthalmic Center, as previously described [20]. This project was approved by the Ethics Committee of the Zhongshan Ophthalmic Center, and followed the tenets of the Declaration of Helsinki. Briefly, the anterior segment, vitreous and neurosensory retina were removed and an eye cup was made. The RPE cells were immersed in a trypsin (0.05%)-EDTA (0.02%) solution at 37 °C for 1 h. Culture medium with 20% FBS was added, and the RPE were isolated and collected with a pipette, using a dissecting microscope. Isolated cells were centrifuged, resuspended and seeded to Corning culture plates (in Dulbecco’s modified Eagle medium (DMEM) containing 10% fetal bovine serum, penicillin (100 µ/µl), streptomycin sulfate (100 mg/ml), and 2mM L-glutamate in. Experimentation was performed using 70%–80% confluent cells at cell passage 3 to 8.

*Flow cytometry:* Confluent RPE cells were stimulated with 100 nM PMA, 100 nM thymeleatoxin and DMEM (as control group) and collected at each time point, then incubated in PBS (8.00 g/l sodium chloride, 0.20 g/l potassium chloride, 1.56 g/1 Na\(_2\)HPO\(_4\)·H\(_2\)O, 0.20 g/l KH\(_2\)PO\(_4\) containing 50 µg/ml/10⁶ cells RNase A and 50 µg/ml/10⁶ cells propidium iodide (PI) for 30 min at 37 °C. The cell cycle analysis of treated cells at each time point were done on FACScan Flow Cytometer (Becton Dickinson). The red fluorescence (PI) from cells were excited at 488 nm using channel 2. The distribution of cells in different phases of the cell cycle was obtained by analyzing fluorescence intensities with Lysis II software (Becton Dickinson).
DMEM and allowed to grow to confluence. Then the cells were incubated with 100 nM thymeleatoxin for 24 h, or transfected with 100 nM siRNA using lipofectamine 2000 according to the manufacturer's protocol, with some modifications. The cells were fed with transfection reagent in serum-free DMEM for 24 h. Three independent siRNAs

### Table 1. Primers and PCR Conditions of Cell Cycle Regulator Genes

| Name   | Sequence               | Product size (bp) | Tm (°C) |
|--------|------------------------|------------------|---------|
| CDK1   | F: TTTTCAGAGCTTTGGGCACCT  
         | R: CCATTTCAGGAAATATCGT  | 195     | 55      |
| CDK2   | F: CATCAGTCGAGGATGGCTGCT  
         | R: CAGAGACTCCAAAGAGCAG  | 173     | 57      |
| CDK3   | F: TTTGCAGAGATGGGTACACTG  
         | R: AGTCCCTTCCTGGTCCACTT  | 167     | 57      |
| CDK4   | F: GAAACGCTGAAAGCCGACAG  
         | R: AGGCAGAGATCCGCTTGTG  | 213     | 57      |
| Cyclin A | F: TTAATTCGACCTTGAGATG     
          | R: CTCTGGGCTGGTGAAGAGAG  | 224     | 55      |
| Cyclin A1 | F: ACCCCAAAGGATGGAGTGTG     
           | R: GGAAGCCATTTTTTCTGAACT  | 198     | 55      |
| Cyclin B1 | F: CCGGAAGTCTGAGGAAACT     
            | R: AACATGAGCAGTGACCAA  | 177     | 55      |
| Cyclin B2 | F: TGTCAGTCGAGGAAACTC     
           | R: GAAAGCAGAGTACGAGCAAT  | 218     | 55      |
| Cyclin C | F: AGGCCCAAGTCTTATGTCTTCT   
         | R: TGTTGAAACCCCTCTCTAC  | 231     | 59      |
| Cyclin D1 | F: AACACTCGTGCAGCGCTTCCT     
           | R: CCAGTTAGTTGGCTGACCCA  | 204     | 57      |
| Cyclin D2 | F: TGGGGAAGTGGAGATGGAC   
           | R: ATGACTAGACGGTGGATCATC  | 175     | 57      |
| Cyclin D3 | F: TGGAGTCTGAGGATGATG     
            | R: TGCACCAGTTTTTCTGATGTC  | 190     | 55      |
| Cyclin E1 | F: CAGATTTCGACAGCCTTGGAG   
            | R: TCCCCGTCTCCTTATAACC  | 225     | 57      |
| Cyclin E2 | F: CAGGTTCGAGCTTGGGACAGT    
            | R: CTCCATCCGACACTGGTAC  | 199     | 59      |
| P16    | F: CTCTGAGGACGAGCTTGC      
         | R: CATTCCTCTTCTCTTTGGCTT  | 158     | 57      |
| P18    | F: TGCCAACTGGGATTTGGGAA    
         | R: GGCAAGTTCCCTCTTATTAT  | 223     | 51      |
| P19    | F: CTGCGAGTCTATGATTTGGG    
         | R: CAGCAGTGACCTCCTTTGA  | 229     | 57      |
| P27    | F: ATGTCACAGCTGGAGGTGCT    
         | R: TCTCTGCAGTTGCTCTTCAAA  | 152     | 57      |
| P21    | F: GACACCCTGGAGGTTGACCT    
         | R: CAGGTTCACATGCTTCTTCTT  | 172     | 59      |
| P107   | F: CCAGTTGGTGCTGCAATCAG    
         | R: GAACAGCAGAGTATGGAGAG  | 164     | 59      |
| Rb     | F: GGAAGCAGAAGCCCTCCTAAC  
         | R: TTTTCTGGTTGGCTATTCG    | 153     | 57      |
| GAPDH  | F: ACCCCAGAAGCTGAGGATGG    
         | R: TGCTCTGAGGCAATAACCTTGG  | 415     | 55      |

RNA (5 µg) was reverse-transcribed following the protocol of the SuperScript™ first-strand synthesis system. cDNAs encoding the cell cycle regulator genes were amplified by PCR as follows: denaturation for 30 s, annealing for 30 s and elongation at 72 °C for 60 s. Primer sequences were designed using Primer 3.
directed against PKCα (A1–A3) were used, along with one control of scrambled siRNA (C): A1, dTdT Ggc ugc uag cag cau ucu uu; A2, dCd Acc uac guu caa cga au; A3, dTd Acc gca caa cau acu uu; C, (product #2005527113152; Ruibo Biotech, Guangzhou, China). The medium containing thymeleatoxin or siRNA-PKCα was removed 24 h later.

**Immunofluorescence analysis:** Human RPE cells grown on coverslips were stimulated for 24 h with 100 nM PMA, 100 nM thymeleatoxin, and 100 nM siRNA-PKCα. They were fixed for 15 min in PBS containing 4% paraformaldehyde, and then rinsed three times in PBS. All reagent incubations were performed in a humidified chamber. The primary antibodies were incubated for 16 h in a solution of PBS at room temperature; horse serum was used as the negative control instead of the primary antibody. After washing four times for 10 min in PBS, FITC and Cy3 -labeled secondary antibodies were incubated for 40 min at 37 °C. Then Hoechst 33342 was incubated for 5 min at room temperature. After three rinses in PBS, coverslips were mounted onto glass slides. Slides were analyzed on a Zeiss laser scanning confocal microscope (LSCM510META). Each antibody was used in a minimum of three separate experiments.

**Data and statistical analysis:** Results are expressed as mean ±standard deviation (SD). Statistical analyses were performed upon comparisons using one-way ANOVA (ANOVA). A value of p<0.05 was considered significant.

**RESULTS**

**PKC activation with a phorbol ester affects cell cycle progression:** Previous work has shown that the PKC inhibitors, hypericin, and calphostin C, dramatically affect the growth rate of RPE cells [9,10]; however, the identity of the PKC isoform involved has remained unclear. To establish which isoform of PKC is potentially involved, we tested the effect of a phorbol ester, PMA, a potent activator of conventional and novel PKC isoforms, on the cell cycle progression of human RPE cells. As shown in Figure 1A,B, after 3 h following the addition of 100 nM PMA, RPE cells entered the S phase. The numbers that entered into S phase at 6, 9, and 12 h time points in PMA-treated RPE cells were decreased when compared with those in the control cells (p<0.05). In contrast, the numbers that entered into the G2-M phases of the cell cycle were increased between 3 and 12 h of treatment, indicating that PMA can slightly affect progression through the cell cycle. By 24 h of treatment, the distribution of the cells between the different phases of the cell cycle was similar to that of the control cells treated only with the vehicle dimethyl sulfoxide. However, control cells showed no significant change in distribution between the different phases of the cell cycle during the 24 h time course examined.

To further confirm that cells were not blocked in the G2 phase, PI-stained RPE cells grown on glass coverslips were analyzed by immunofluorescence microscopy at various times following PMA or vehicle treatment. Cells at all stages of mitosis could be observed in both PMA-treated cells and vehicle-treated cells, indicating that the cells were progressing normally through mitosis (Figure 1C). Therefore, PKC activation seemed to play a role in the regulation of cell cycle progression.

To identify the isoform of PKC responsible for the progression of the cells through the cell cycle, we monitored the effect of PMA on the subcellular localization of the nine PKC isoforms expressed in human RPE cells. Translocation of PKC from the cytosol to the membrane is a hallmark of its activation [25]. Upon PMA treatment, only PKCα and PKCδ were translocated from the cytosolic to the particulate fraction. PKCβII, PKCβI, PKCε, PKCζ, PKCλ, PKCθ, and PKCι were not affected by PMA (Figure 2). PKCα was completely downregulated by proteolytic degradation by 6 h of treatment, while PKCδ was translocated from the cytosolic to the particulate fraction between 3 h to 24 h of treatment. Hence, the data indicate that of the nine PKC isoforms expressed in RPE cells, only PKCα and PKCδ were significantly activated by PMA stimulation.

**PKCα is necessary and sufficient to affect progression through the cell cycle:** To differentiate between PKCα and PKCδ, we used the conventional isoform-specific PKC agonist thymeleatoxin [26]. Since PKCα is the only one of the three isoforms that translocates, it is likely that this agonist would only affect PKCα.

Flow cytometry analysis of 100 nM thymeleatoxin-treated RPE cells (Figure 3A) showed a cell cycle progression profile similar to that obtained with PMA (Figure 1A). Western blot analysis confirmed that PKCα was specifically translocated (and activated) by thymeleatoxin, whereas PKCδ remained unaffected (Figure 3B).

Further confirmation of the specific role of PKCα in the regulation of the cell cycle progression of RPE cells was provided by PKCα depletion experiments. RPE cells were pretreated with PMA for 48 h to deplete the cells of their endogenous PKCα. Cells were then restimulated with 100 nM PMA and 100 nM thymeleatoxin, and their distribution between the different phases of the cell cycle was analyzed between 0 and 30 h following restimulation by flow cytometry (Figure 3C). In the absence of a detectable level of PKCα, there was no significant change in the cell cycle progression of RPE cells following PMA or thymeleatoxin stimulation over the 30-h time course (Figure 3C), unlike the case of cells containing PKCα (Figure 1).

Although PKCα activation affects cell cycle progression, the proliferation of the PKC isoform involved has remained unclear. The role of PKCα in cell proliferation was further addressed using siRNA or thymeleatoxin. Equally seeded cultures were grown and counted, giving a direct reading of their growth rate. SiRNA-PKCα clones exhibited a growth rate of about half the rate of the control cells (Figure
Figure 1. PKC activation with PMA affects cell cycle in human RPE cells. Flow cytometry analysis of PMA-treated RPE cells (A) shows decreased S phase and increased G2-M phases cell numbers when compared with that of untreated RPE cells (B). For each side scatter plot, the y-axis is the number of cells, while the x-axis is the DNA content. Values from each scatter plot are graphed below panels A and B. Similar results after PMA treatment were obtained in eight independent experiments. C: Immunofluorescence of cellular DNA stained with propidium iodide (PI) showed cells in interphase or at different stages of mitosis. RPE cells were grown on glass coverslips for 24 h, treated either with PMA or with thymeleatoxin for 9 h, and then fixed.
3D), thus indicating that PKCα levels are directly proportional to the basal proliferation rate of RPE cells. However, PKCα agonist, thymeleatoxin, did not exhibit a growth rate of the RPE cells (Figure 3D), indicating that thymeleatoxin has no significant effect on cell proliferation. Altogether, the data strongly suggest that PKCα-specific activation is necessary and sufficient for the regulation of human RPE cells through the cell cycle. Moreover, our data indicate that PKCα affects RPE cell proliferation, since decreased PKCα expression correlates with decreased proliferation.

\( p27^{kip1} \) mRNA and protein levels are downregulated following PKCα activation: As shown in Figure 4A, PKCα can be downregulated at the 3-h time point following 24 h of siRNA-PKCα treatment, but not upregulated following PMA and thymeleatoxin treatment. To evaluate the molecular mechanism by which PKCα induces cell cycle progression, we used RT–PCR to examine the transcript levels of cell cycle-
Figure 3. PKCα is necessary and sufficient to affect cell cycle progression. **A**: Flow cytometry analysis of RPE cells after 100 nM thymeleatoxin treatment shows a cell cycle progression profile similar to that obtained with PMA in eight experiments. **B**: Western blot analysis shows that PKCα was rapidly translocated to the membrane by thymeleatoxin and downregulated within 24 h, the protein remained undetectable after 48 h of treatment, however, PKCδ was not translocated and was not downregulated at all time points. Eighty micrograms of protein was loaded in each well. Optical density of PKCα determined by densitometric imaging is shown (Mean±SD, n=3). The β-actin band with 42 kDa is used for quantitation. **C**: Flow cytometry analysis of RPE cells shows that there was no significant change in the cell cycle progression following PMA or thymeleatoxin restimulation when compared with the control over the 30 h time course after 48 h of PMA treatment. **D**: PKCα activity regulates the growth rate of RPE cells. Approximately 110,000 RPE cells were seeded and then incubated with thymeleatoxin or siRNA-PKCα for 24 h. The numbers of cells were counted using a Coulter Counter and displayed in the top panel (* p<0.0001). Western blot using an anti-PKCα antibody showed that the total PKCα level was dramatically decreased in siRNA-PKCα treated cells; 40 µg of protein was loaded in each well.
regulatory proteins after 3 h of treatment. We found that p27 mRNA was obviously downregulated following PMA or thymeleatoxin treatment, and upregulated following siRNA-PKCα treatment. Levels of other mRNA (CDK1, CDK2, CDK3, CDK4, Cyclin B1, Cyclin B2, Cyclin D2, Cyclin D3, Cyclin E1, Cyclin E2, p16, p18, p21, and Rb) remained unaffected by PKCα activation. The mRNAs for CyclinA1, CyclinC, CyclinD1, p19, and p107, could not be detected in this assay (Figure 4B). During 24 h of stimulation with PMA or thymeleatoxin, p27 mRNA was strongly and rapidly downregulated at 1, 2, and 6 h following PMA treatment, or at 1 h and 2 h following thymeleatoxin treatment (Figure 4C,D).

Consistent with a change at the mRNA level, the p27 protein was also downregulated over a 24-h period following PMA or thymeleatoxin treatment of RPE cells (Figure 5). In untreated RPE cells, the p27 protein level remained constant, while in cells treated either with PMA or thymeleatoxin, p27 was strongly downregulated at the 1 h and 3 h time points. These data indicate that p27kip1 is the only cell cycle-regulatory molecule downregulated following PKCα activation.
Immunofluorescence colocalization of PKCα and P27:

Confocal microscopy clearly showed that the cultured RPE cells formed monolayers with typical polygonal cellular arrays. As shown in Figure 6, PKCα and p27 have obvious cytoplasmic localizations and slight nuclear localization, and mostly colocalized in the cytoplasm of the cells. Although minor staining differences appeared among 100 nM PMA, 100 nM thymeleatoxin, and 100 nM siRNA-PKCα when compared with that of control group, PKCα and p27 seemed colocalized in the cytoplasm of the cells with decreased cell numbers in siRNA-PKCα.

**DISCUSSION**

Effect of PKCα on the cell cycle progression in RPE cells: We have found that PKC activation by phorbol esters affected RPE cell progression through the cell cycle. This was consistent with previous data showing the correlation between the results on PKC activity and RPE cell proliferation [10] and the animal results that demonstrated inhibitors of PKC hypericin could have efficacy in rabbits with PVR [16,17]. Moreover, we have determined that only PKCα activation is necessary and sufficient to regulate cell cycle progression of RPE cells, and that the expression level of PKCα correlates with the proliferation of RPE cells.

It is well known that PKC has been associated with the regulation of cell cycle progression either during the G1-to-S progression or during the G2/M transition [27-29]. PKC has been shown to regulate G1 progression through the modulation of CDK activity, either by modifying cyclin or CDK expression levels, or by modifying the expression of the cyclin-CDK inhibitors. Due to the relevance of PKC isozymes in the control of cell cycles, both in G1/S and in G2/M, the elucidation of such complex intracellular networks using cellular and animal models has become of the outmost importance.

Predominantly, PKC plays an inhibitory role in many cell cycle progressions [23,30,31]. In intestinal epithelial cells, for instance, PKCα-specific activation resulted in G1 arrest and delayed transit through the S and G2/M phases through an upregulation of p21 and p27, resulting in hypophosphorylation of Rb [23]. However, in contrast to most cell types, phorbol esters accelerated growth factor-induced Swiss 3T3 cell cycle entry and progression into the S phase by elevating cyclin D1 levels and downregulating p27Kip1 expression [32]. In human RPE cells, ethambutol may exert toxic effects in RPE, including the suppression of cell growth, formation of cytoplasmic vacuoles, and reduction of phagocytic functions via the PKC signal pathway [33]. Alkylphosphocholines inhibit proliferation of RPE cells and RPE-mediated matrix contraction in vitro at nontoxic concentrations through the inhibition of PKC activity [34].
Figure 6. Confocal images of p27 and PKCα colocalization in RPE cells. PKCα and p27 have obvious cytoplasmic localizations and slight nuclear localization, and mostly colocalized in the cytoplasm of the cells stimulated with PMA, thymeletaxin, and siRNA- PKCα. PKCα (FITC, green label) p27 (Cy3, red label), nuclei (Hotchest 33342, blue label), PKCα, and p27 colocalization (yellow label).
Our study is the first to show that the activation of PKC, and specifically PKCα, exerts effects on the S to G2/M progression of the human RPE cell cycle, as shown in Figure 1A and Figure 3A, and that the inhibition of PKC decreases the proliferation, as shown in Figure 3D. Recently, aprinocarsen, an antisense oligonucleotide (ASO) against PKCα, has been used to decrease the malignant proliferation in clinic trials in different cancers [35-39]. Similarly, since PKCα was the only isoform associated with the proliferation of RPE cells in our study, it may be a rational approach for targeted therapies against RPE cell proliferation and PVR disease.

**Downregulation of p27kip1 following PKCα activation in RPE cells:** To elucidate the mechanism by which PKC increased the RPE cell cycle progression, we analyzed the expression of various cell cycle-regulatory proteins following PKC activation. We found that the only cell cycle regulatory protein downregulated by PKCα activity was the inhibitor p27kip1, which has been proposed to be part of a cell-intrinsic timer that arrests the cell cycle and initiates differentiation in several lineages [40,41].

Several groups have reported gigantism and multiple organ hyperplasia in mice with targeted disruption of the p27kip1 gene [42-47]. Some of the most dramatic phenotypic changes in these animals were involved in the retina. For example, Nakayama et al. reported that the RPE exhibited an increase in thickness in its apical to basal dimension compared to that seen in the congenic C57BL/6J strain [44]. Other results showed that the neural retina exhibited focal areas of dysplasia, attributed to extended histogenesis of photoreceptors and Müller cells and to the displacement of reactive glia into the layer of photoreceptor outer segments, leading to a disruption in the normal organization of the outer nuclear layer [45,46]. Defoe et al. examined the retinas of p27kip1 knockout mice in more detail and not only found that p27kip1 was an important factor in regulating RPE proliferation during development, but also observed that this protein may be a crucial factor involved in generating appropriately polarized epithelial cells and in the construction of the photoreceptor-RPE interface [47]. From these results, it was concluded that p27kip1 downregulation may be involved in the process of RPE cell proliferation and PVR disease. In our study, p27kip1 was downregulated by PKCα activation at the gene and protein levels in RPE cells. Moreover, p27kip1 and PKCα colocalized within the cells, as shown in Figure 6. Therefore, inhibitors of PKCα could have antiproliferative effects on RPE cells in vitro and as a potential therapeutic drug for PVR via p27kip1 downregulation. In addition, a possible role of PKCα in cell cycle progression and proliferation in RPE cells should be ruled out in the future study.

On the other hand, several studies have reported the PKC-induced upregulation of p27 in other cell types [48-50]; however, this was associated with cell cycle blocks, unlike the case for RPE cells reported here. Taken together, the relationship between PKCα, p27kip1, and PVR is illustrated in Figure 7.

It is well known that PVR is a result of various biologic reactions, such as the synthesis of the extracellular matrix, contraction of membranes, and apoptotic change of photoreceptors. Of all the cells involved in PVR, the RPE cell is a central player, but the inhibition of RPE cell proliferation is not sufficient to inhibit PVR, which has been proven by studies of anticancer drugs for PVR since the 1990s.

In summary, we have found that PKCα affects the cell cycle progression and proliferation in RPE cells through the downregulation of p27kip1. These results suggest that PKCα can be used as a potential therapeutic target against RPE cell proliferation and PVR disease.

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