·Basic Research·

The changes of protein kinase C for human retinal pigment epithelium and retinal glial cells proliferation induced by the subretinal fluid

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Abstract

• AIM: To study the effect of the subretinal fluid (SRF) on proliferation of retinal pigment epithelium (RPE) cells and retinal glial (RG) cells and associated activation and translocation of protein kinase C (PKC) as well as the application of PKC inhibitor.

• METHODS: RPE and RG cells were disintegrated to obtain PKC activity of cytoplasm and cellular membrane after being treated by the subretinal fluid (SRF) from the different stages of PVR patients (grade B and C) or being treated with PKC specific activator [phorbol-12-myris-tate-13-acetate (PMA)] or normal vitreous or DMEM culture medium. PKC activity in cytoplasm and cellular membrane was measured using radioactive isotope ^{32}P labeling in a specific reaction of phosphorylation on PKC substrate. In addition, the PKC inhibitor, dequalinium chloride, was used to pretreat the RPE and RG cells before the cells exposed to SRF or PMA or normal vitreous. ^{3}H-TdR (tritiated thymidine) was used to measure the levels of proliferation of RPE and RG cells with or without the activation and translocation.

• RESULTS: SRF and PMA promoted the proliferation of RPE and RG cells. SRF and PMA activated PKC in the cytoplasm of RPE and RG cells and the activated cytoplasm PKC translocated to the cellular membrane of RPE or RG cells. The cell proliferation or PKC activation or translocation was not equally active in RPE as in RG cells. However, PKC inhibitor which attenuated the cell proliferation did not show significant difference on inhibition of RPE and RG cell proliferation($P>0.05$).

• CONCLUSION: SRF can lead to the activation and translocation of PKC in RPE and RG cells, which promote the proliferation of RPE and RG cells. Dequalinium chloride can inhibit PKC activation and translocation hence slow down the cells proliferation.

• KEYWORDS: subretinal fluid; protein kinase C; retinal pigment epithelium cell; retinal glial cell; PKC activation and translocation; cell proliferation

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INTRODUCTION

The development of proliferative vitreoretinopathy (PVR) has been attributed to the migration and multiplication of RPE and RG cells [1]. This proliferating process is closely correlated with disturbance of intracellular regulation of RPE and RG cells[2]. Protein kinase C (PKC) is an essential component of intracellular regulation signaling pathways which plays a critical role in the course of cell proliferation by affecting phosphorylation of residues of targeting serine and threonine proteins and by controlling the level of the genetic transcription[3,4]. The increased activity of PKC was indicated by the increased level of PKC expression in cellular membrane [5]. The majority of PKC function have been obtained by studying the tumor cell proliferation and not much efforts have been put on the mechanisms by which PKC increases [6,7]. The roles of activity of PKC on how RPE and RG cells converting from the steady state to the higher vegetative state which relates to the development of PVR, have not been well explored. The current studies were undertaken to observe whether RPE and RG cells can procure to proliferate when exposed to subretinal fluid, and how their proliferation relates to the activity of PKC, and whether PKC inhibitor can hinder the RPE and RG cells' multiplication or proliferation.

MATERIALS AND METHODS

RPE and RG Cell Culture Donor human eyes (donors aged 20 and 30 years) were obtained without their cornea from Department of Ophthalmology of the First Affiliated Hospital of China Medical University. RPE and glial cell lines were established using the culture techniques described previously[8,9].

Collection of Subretinal Fluid and Normal Vitreous Subretinal fluid was collected from 24 patients (average age

of 43.8 ±6 years) with rhegmatogenous retinal detachment during the sclerotic cingulum operation, including B grade 12 and C grade 12 (The grading was based on Cosmo-Institute of Retinopathyclass A, B and C). At least 1mL of subretinal fluid was collected and preserved below minus 70℃. The normal vitreous came from the eyes of donor who died within 4 hours before vitreous sampling.

Groups of Experiment There were five experimental groups: G_0 (100mL/L CS+DMEM+NS) blank control, G_1 [50% Corporis vitre+50% (DMEM+100mL/L CS)] positive control, and G_2 (PMA,100nmol/L), G_3 (Disposal groups): G_{3B} [50% SRFB+50% (DMEM+100mL/L CS)] and G_{3C} [50% SRFc+ 50%(DMEM+100mL/L CS)] and G_4 [PKC inhibitor dequalinium chloride (0.5μmol/L)] were the study groups. Groups of $G_{3\ (C,B)}$ and G_1 were divided respectively into two sections. One was to detect activity of PKC of RPE and RG cells; the other section was to determine level of cell multiplication. Time of being observed was from zero to 24 hours.

To Determine Level of Cell Proliferation Some bibasic RPE and RG cells which derived from an eye were prepared respectively into cell suspension, and then cells were adjusted to 1×10^9/L and inoculated in 24-well plates with the culture medium (100mL/L CS+DMEM) in an incubator with a humidified atmosphere of 50mL/L CO_2, 950mL/L air at 37℃ for 24 hours. RPE and RG cells were stimulated by additives as indicated by the groups (G_0, G_1, G_2, G_{3B}, and G_{3C}) when the cells were in adherent state. The culture medium was blotted before addition of the culture fluid containing ^{3}H-TdR. After RPE and RG cells had been incubated for 24 hours and the medium was again. Another medium blotting was performed after two times of PBS (pH7.0) wash. DNA of RPE and RG cells was extracted through 1mol/L NaOH. Radioactivity of specimens was measured on Beckman scintillation counter. One hole was repeated three times and the mean was used.

To Extract PKC of Cytoplasm and Plasma Some bibasic RPE and RG cells were also prepared respectively into cells suspension, and then cells were adjusted to 1×10^9/L and inoculated in 24-well plates in an incubator with a humidified atmosphere of 50mL/L CO_2, 950mL/L air at 37℃ for 24 hours. RPE and RG cells were stimulated by G_0, G_1, G_2, G_{3B}, and G_{3C} respectively at different time when the cells were being on adherence. After the predetermined experiments on those cells were done, the stimulating fluids were removed and enzyme reaction was terminated. The RPE and RG cells were rinsed twice within two minutes and the cells were collected respectively. The plasmosin extract 500μL was added into the cell collection which then subjected to ultrasonic fragmentation for four times (each times for 15 seconds, intensity at 25%) in iced bath at 0℃. The resultant cells homogenate was centrifuged at 20 000r/min for 15 minutes at 4℃. We obtained primary PKC extracts of cytoplasm by collecting supernatant containing endochylema. The residual sediments were added membrane protein extract, and then were shaken 50 minutes at 4℃. Sequentially, the samples were centrifuged again at 20 000r/min for 15 minutes at 4℃, and primary PKC extracts of plasma membrane was obtained by collecting the supernatant containing plasma membrane and were stored under minus 70℃ for further analysis.

PKC Inhibitor Stimulation RPE and RG cells were pretreated by dequalinium chloride (final concentration 0.5μmol/L) for 20 minutes after the cells reach adherence. Sequentially, dequalinium chloride was removed and RPE and RG cells were stimulated with all experimental groups except G_4. Then the level of cell proliferation and PKC extraction were performed as described above.

Determination of Activity of PKC 200μL of determining solution [containing 10mmol/L $MgCl_2$, 0.5mmol/L$CaCl_2$, 25mmol/L Tris-HCl (pH7.2), 25μmol/L MBP, 0.1g/L diacylglyceryl-phosphorylserine, 0.025g/L diacylglycerol] was added to the extracts of cytoplasm PKC and γ-^{32}P-ATP (37kBq/200μL). Sample was first put in warm bath with 37℃ for 5 minutes, and then it was placed in iced bath and 150mL/L TCA was added to terminate the reaction. Every test tube with enzyme preparation was repeated to measure for three times to get mean of the data. The extracting of plasma membrane PKC was also performed. The acid sediments that produced from the above procedures were collected to XtalScint's micropore films, and then were rinsed by 100mL/L TCA for two times. Sequentially, they were vacuum-filtrated to pass XtalScint's micropore films. Liquid scintillation counter was used to determine radioactive square pulse blink value in per minute (cpm) and spectro-photometer (wavelength 650nm) was used to measure their protein level. Enzymatic activity of PKC was calculated according to the instruction from the manufacturer and unit was demonstrated by means of per minute, per mg zymoprotein transferred pmol phosphate radical (pmol^{32}P•min^{-1}P•mg^{-1}) at 37℃. The activity of PKC of cytoplasm and plasma membrane was recorded respectively[10,11].

Statistical Analysis All analysis were performed with the SPSS 10.0 statistical package and all results were presented as mean±standard error of the mean(SEM). Interclass analyses of difference were performed with analysis of variance and two sets were performed with independent-samples t tests. Coefficient correlation about SRF was calculated as well.

RESULTS

Proliferative Levels of RPE Cells The value of CPM of ^{3}H-TdR of RPE cells that were treated by G_0 demonstrated a

torpid ascending tendency during four hours, at the fourth hour, it increased obviously, and reached and retained on its crest-time from the tenth hour to forever. There was no change about the CPM of RPE cells in G_1. The CPM of RPE cells started to increase remarkably at the first hour in G_2, then at the fourth hour, the value of CPM reached and retained on its crest-time to forever. The remarkable increase was at the fourth hour in G_{3B} and G_{3C}, ten hours later, reached its crest-time and retained on its crest-time to forever, but their high-peaks were different. The high value of CPM in G_0 group was lower than that in G_{3B}, G_{3C} and G_2, and the high value in G_2 was lower than that in G_{3B} and G_{3C}. The value of CPM at its high-peak in G_{3B} group is lower than G_{3C}. Interclass ANOVA analyses showed F=11.36, P<0.05. G_0, G_{3B}, G_{3C} and G_2 could excite RPE cells to proliferate. After RPE cells had been pretreated by G_4 for 20 minutes, the value of CPM of ^{3}H-TdR for other experimental groups' RPE cells no longer increased, even if RPE cells were exerted by every group stimulating factor again, and all data from all experimental groups was close to each other, and their curve overlapped into one (Figure 1).

Proliferative Levels of RG Cells All experimental groups except G_4 could excite RG cells to proliferate. Being different from RPE cells, there were variances from the values of CPM and at point of the moment to step up obviously and the point of occupied crest-time, and G_1 could also procure RG cells to proliferate (Table 1).

Variances of Activity of PKC in RPE Cells There were no changes for activity of PKC in RPE cells in G_0 and G_1. In G_2, the activity of cell membrane PKC demonstrated a torpid ascending tendency in 10 minutes, sequentially, it increased obviously, and reached its crest-time at the fortieth minute after RPE cells were stimulated, meanwhile, the activity of kytoplasm's PKC gradually decreased with allelism at same time. Comparing G_{3B} and G_{3C}, the activity of membrane PKC started to increase gradually in beginning of 20 minutes after RPE cells were stimulated, sequentially, it increased obviously, and reached its crest-time at the sixth hour in G_{3B} and at the third hour in G_{3C}. The activity of kytoplasm's PKC began to decrease gradually with allelism in G_{3B} and in G_{3C}. In G_{3B}, the activity of membrane PKC began to decrease gradually when the activity of cell membrane PKC of RPE cells reached their crest-time; but in G_{3C}, after the activity of cell membrane PKC remained at crest-time for three hours, the activity of membrane PKC began to decrease gradually. In spite of the same changing tendency, the corresponding numerical value was lower in G_{3B} than in G_{3C}. The data of G_{3B} and G_{3C} were performed with independent-samples $t$tests. Its result showed t=2.127, P<0.05. The variances of PKC of membrane which derived from RPE cells excited by SRF demonstrated positive correlation to the grading of

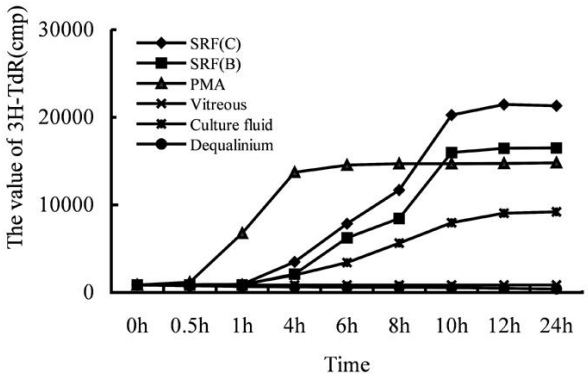

Figure 1 RPE cell´s value of ^{3}H-TdR treated by different agent. After G_0, G_1, G_2, G_{3B} and G_{3C} were pretreated by dequalinium chloride, their growth curves overlapped into one. Independent-samples t tests were performed with G_1 and G_4, P>0.05, and all the rest two sets results were P<0.05

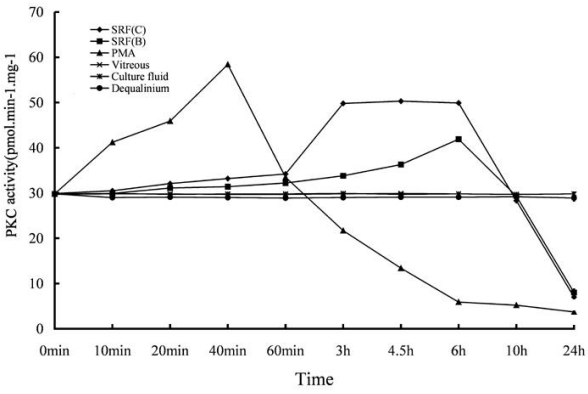

Figure 2 The activity of m-PKC of RPE cells treated by different agent. Curves of G_0 and G_1 overlap. After G_0, G_1, G_2, G_{3B} and G_{3C} were pretreated by dequalinium chloride, their growth curves overlapped into one. Independent-samples t tests were performed with G_0, G_1 and G_4, P>0.05, and all the rest two sets results were P<0.05

PVR. The higher rank of PVR was, the larger effect SRF irritated RPE cells on the changes of PKC, and coefficient correlation was 0.8472. There were descending variances of activity of cell membrane PKC in G_2, G_{3B} and G_{3C} after crest-time, finally, the activity of cytomembrane PKC descended to below primary level. ANOVA was performed with data of all experimental groups except G_4, and F=5.714, P<0.05. After RPE cells had been pretreated by G_4, activity of kytoplasm and plasma membrane PKC for other experimental groups' RPE cells no longer varied and retained primary level even if RPE cells were exerted by every group stimulating factor again, and all data from all experimental groups were close to each other, and their curves overlapped into one (Figure 2).

Variances of Activity of PKC in RG Cells Being different from RPE cells, the activity of cell membrane PKC reached and remained still at crest-time at the moment of the fifth hour in G_{3B} and the third hour in G_{3C}, and

Table 1 The value of CPM of ^{3}H-TdR of RG cells treated by different agent (mean ± SEM)

Disposal factors	The value of CPM in disposal time(h)								
	0	0.5	1.0	3.0	5.0	7.0	9.0	12.0	24.0
Culture fluid	854±37	864±38	923±42	1874±28	3327±94	5439±117	7787±223	8964±321	9115±328
Vit	852±37	859±37	854±36	854±37	856±38	853±36	852±37	861±38	858±37
PMA	855±37	1173±54	6752±193	12738±386	13549±392	13682±403	13713±418	13754±426	13827±439
SRF(B)	851±36	874±39	882±44	2193±89	6124±177	8353±281	14992±436	15476±487	15495±489
SRF(C)	849±36	895±43	940±47	3278±124	7365±251	10694±419	19261±597	20475±608	20595±613
Inhibitor	854±36	857±37	853±38	856±39	858±36	855±36	854±35	859±38	859±38

After G_0,G_1,G_2, G_{3B} and G_{3C} groups were pretreated by dequalinium chloride, their values were similar to each other. Independent-samples *t* tests were performed with $G_1$and G_4, $P>0.05$,and all the rest two sets results were $P<0.05$. ANOVA was performed with interclass, $F=13.77$, $P<0.05$

Table 2 The activity of PKC of RG cells treated by different agent (pmol·min^{-1}·mg^{-1}, mean ± SEM)

Disposal factors	PKC	Disposal time(min)									
		0	10	20	40	60	180	270	360	600	1 440
SRF(C)	m-PKC	23.9 ± 2.8	28.5 ± 2.9	30.1 ± 3.1	31.2 ± 3.2	34.2 ± 3.3	42.7 ± 4.9	43.3 ± 5.1	42.5 ± 4.8	21.3 ± 2.4	7.0 ± 1.6
	c-PKC	15.3 ± 1.8	15.3 ± 1.7	14.6 ± 1.5	14.1 ± 1.5	13.8 ± 1.3	9.2 ± 1.0	8.1 ± 0.9	6.6 ± 0.7	6.1 ± 0.8	5.3 ± 0.4
SRF(B)	m-PKC	23.8 ± 2.9	26.6 ± 3.0	29.2 ± 3.2	29.5 ± 3.1	32.3 ± 3.2	38.7 ± 4.4	39.1 ± 4.5	38.4 ± 4.3	21.6 ± 2.1	7.1 ± 1.7
	c-PKC	15.2 ± 1.9	15.3 ± 1.8	14.9 ± 1.6	14.5 ± 1.6	14.1 ± 1.4	10.3 ± 1.1	8.6 ± 1.0	6.8 ± 0.8	6.3 ± 0.7	5.7 ± 0.6
PMA	m-PKC	23.8 ± 2.8	36.2 ± 3.7	42.9 ± 3.9	53.4 ± 4.7	33.5 ± 3.8	21.7 ± 2.5	13.4 ± 1.9	5.9 ± 0.9	5.2 ± 0.4	3.7 ± 0.2
	c-PKC	15.2 ± 1.7	6.8 ± 0.9	5.2 ± 0.6	4.7 ± 0.6	3.5 ± 0.4	2.3 ± 0.2	2.0 ± 0.2	1.6 ± 0.1	1.4 ± 0.1	1.1 ± 0.1
Vit	m-PKC	23.7 ± 2.8	23.8 ± 2.8	23.8 ± 2.8	29.7 ± 2.8	23.7 ± 2.9	23.9 ± 2.7	23.8 ± 2.8	23.7 ± 2.9	23.6 ± 2.9	23.7 ± 2.9
	c-PKC	15.3 ± 1.8	15.4 ± 1.6	15.2 ± 1.9	15.2 ± 1.8	15.3 ± 1.9	15.4 ± 1.8	15.3 ± 1.7	15.2 ± 1.8	15.3 ± 1.8	15.2 ± 1.9
Culture fluid	m-PKC	23.8 ± 2.8	23.7 ± 2.9	23.9 ± 2.8	29.7 ± 2.9	23.8 ± 2.9	23.9 ± 2.7	23.9 ± 2.8	23.7 ± 2.8	23.6 ± 2.8	23.6 ± 2.9
	c-PKC	15.4 ± 1.8	15.4 ± 1.7	15.3 ± 1.9	15.2 ± 1.8	15.3 ± 1.8	15.3 ± 1.8	15.3 ± 1.9	15.2 ± 1.7	15.3 ± 1.7	15.2 ± 1.8

Independent-samples *t* tests were performed with G_0,$G_1$and G_4, $P>0.05$,and all the rest two sets results were $P<0.05$. ANOVA was performed with interclass, $F=6.581$,$P<0.05$

independent-samples *t* tests was performed with the data of G_{3B} and G_{3C}, $t=3.051$, $P<0.05$. ANOVA was performed with data of all experimental groups except G_4, and $F=6.581$, $P<0.05$. Coefficient correlation between G_{3B} and G_{3C} was 0.6135. Other aspects were similar to RPE cells (Table 2).

DISCUSSION

The development of PVR is related to RPE and glial cell proliferation which often occur during rhegmatogenous retinal detachment. We think that the relation of SRF to PVR development is worth exploring.

In our study, we measured the DNA synthesis state of RPE and RG cells in S stage by ^{3}H-TdR incorporation to reflect the proliferation condition of RPE and RG cells respectively. In G_0, we conjectured the low level proliferation of RPE and RG cells was due to certain active substances in DMEM culture fluid which triggered a kind or some kinds of cell signal transduction system to involve in proliferation, or the direct action of growth factors produced by unknown stimulation which promoted the hyperplasia. In G_1, there was no hyperplasia of RPE cells. That phenomenon was due to the action of epithelium cell growth chalone-transforming growth factor (TGF-β) secreted by hyalocytes in the normal vitreous. The mechanism of the anti-proliferation effect of TGF-β on RPE cells was that TGF-β blocked the RPE cells proliferation cycle in G_1 stage preventing entering proliferation stage [12,13]. Maybe the action of TGF-β to RPE cells proliferation was probably superior to the influence of signal transduction system along with RPE cells proliferation, or signal transduction system was not activated. However, TGF-β is the growth factor of RG cell, and it can make RG cells proliferate. In G_2, the peak time of CPM of ^{3}H-TdR with RPE and RG cells came earlier than other groups. The reason was that PMA switched on the PKC signaling system specifically and participated in the proliferation process earlier. In G_{3B} and G_{3C}, the occurrence of proliferation at higher level of RPE and RG cells was because SRF contained components such as alkali phosphatase, serine/threonine, Ca^{2+}, vascular endothelial cell growth factor (VEGF), epidermal growth factor (EGF), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF)[14], which promoted proliferation of RPE and RG cells, and meantime SRF activated the intracellular signal transduction system including PKC to induce proliferation of RPE and RG cells. Due to the different amount of SRF in G_{3C} and G_{3B}, the proliferation level of in G_{3C} was higher than that in G_{3B}.

In our study, there was no change of activity of cell membrane and cytoplasm PKC of RPE and RG cells in G_0 and G_1. The first probable reason was that there were no any compounds which could promote the activation of the PKC signaling system of RPE and RG cells. The second one was the stimulating and inhibitive factors taking part in activation of PKC signaling system was in balance in G_0 and G_1.The last one, some components of normal vitreous could resist or inhibit the activation of signaling of PKC. The effect of SRF on the activation of cytoplasm PKC of RPE and RG cells in G_{3C} was stronger than that in G_{3B}. We speculated the different amount of active agents in G_{3C} and in G_{3B} made the concentration of the effective ingredient for activation and translocation in G_{3C} higher than that in G_{3B}. In our study, there was a tendency of activation and translocation of PKC of RPE and RG cells in G_2 differed from that in G_{3C} and G_{3B}. We presume that different mechanism between SRF and PMA for PKC activation and translocation made the RPE and RG cells cultured by SRF (in G_{3C} and G_{3B}) *in vitro* take more time on activation and translocation than in G_2. At present, we have known that some endogenous active compounds that can active PKC such as adenosine, noradrenalin, bradykinin, hyperglycaemia, unsaturated fatty acid, inositol triphosphate (IP3), diacylglycerol (DG), phosphatidylserine (PS) and Ca^{2+}[11,15]. And some polypeptide growth factors such as insulin, EGF, VEGF, PDGF, FGF and interleukin-3 (IL-3) could promote the cells to produce more DG [16-18]. In that case, with Ca^{2+} existing at same time, the kytoplasm PKC will be activated to translocate to cellular membrane, and the activity of PKC will be enhanced [19,20]. It was found that there was activation and participation of the PKC signal system in the proliferation process of RPE and RG cell. As the prolongation of time, the amount of C-PKC was gradually decreased in the proliferation of process of RPE and RG cell, which was induced by SRF, while the amount of membrane PKC was gradually decreased. The CPM of ^{3}H-TdR of RPE and RG cell group gradually ascended in the process that the activity of membrane PKC was increasing. PKC usually exists in an inactive way in the cytoplasm under the condition of physiological environment. It must bind to membrane to be activated [21]. So the PKC signal system took part in the proliferation process of RPE and RG cell after activated is by the way of ascending of m-PKC activity. In the current research the C-PKC, translocated to the cell membrane after activation, took part in and promoted the proliferation of RPE and RG cell by increasing action of phosphorylation to target protein of serine/threonine residue. Our study showed that the degree of RPE proliferation, the activity level of membrane PKC, and lasting time of its high level were proportionally associated, which suggested the up-regulation of membrane PKC was in accordance with cell proliferation. Meanwhile, the activated PKC accumulated to the cell membrane and activated the proteinase. So it would be restrictively hydrolyzed or degraded into micromolecular protein independent of Ca^{2+} and lipid finally. So far, it has been found that there are PKCα, PKCβ, PKCδ and other subunits in human RPE cells [22,23] which are associated with proliferation and differentiation of cells, therefore the activity increasing of PKC has a quite effect on the proliferation of cells[24,25]. When the activity of membrane PKC increase, it would make a series of protein, correlative phosphorylated substrates, enhanced caryomitosis through augmenting Na^+ inflow and H^+ efflux along with the ascending pH value, and ultimately to the increased uptake of the amino acids, which lead to the proliferation of RPE and RG cells [26-28]. The CPM of ^{3}H-TdR of all groups fell behind the activity crest-time of m-PKC for 3-7 hours. It was probably because that the progress from PKC making the substrates phosphorylate to DNA synthesis needs a period of time. Dequalinium chloride is a kind of liposoluble compound, which has a special binding site at the region of accommodation and catalytic domain of PKC, and it has almost the same degree of inhibition to the two regions of PKC. After the RPE and RG cells pretreated by dequalinium chloride for 20 minutes in our study, it competitively inhibited the activation of PKC, which stops the cell in S period, and blocks the proliferation of RPE and RG cells. It suggests that in the proliferation process of RPE and RG cells, activation of PKC signal system play an important role, and the activated PKC take part in the cell proliferation process by the cycle relative to DNA and protein synthesis.

In summary, PKC is an important signaling molecule participating in the process by which SRF induce proliferation of RPE and RG cells. The changes of activation and translocation of PKC of RPE and RG cells is in direct proportion with grades of PVR from which the SRF is collected. Therefore, using a specific inhibitor of PKC can be helpful to prevent RPE and RG cells proliferation in PVR.

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