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EXTENDED REPORT

Selective blockade of phosphodiesterase types 2, 5 and 9 results in cyclic 3',5' guanosine monophosphate accumulation in retinal pigment epithelium cells

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Aim: To investigate which phosphodiesterase (PDE) is involved in regulating cyclic 3',5' guanosine monophosphate breakdown in retinal pigment epithelium (RPE) cells.

Methods: cGMP content in the cultured RPE cells (D407 cell line) was evaluated by immunocytochemistry in the presence of non-selective or isoform-selective PDE inhibitors in combination with the particulate guanylyl cyclase stimulator atrial natriuretic peptide (ANP) or the soluble guanylyl cyclase stimulator sodium nitroprusside (SNP). mRNA expression of PDE2, PDE5 and PDE9 was studied in cultured human RPE cells and rat RPE cell layers using non-radioactive *in situ* hybridisation.

Results: In the absence of PDE inhibitors, cGMP levels in cultured RPE cells are very low. cGMP accumulation was readily detected in cultured human RPE cells after incubation with Bay60–7550 as a selective PDE2 inhibitor, sildenafil as a selective PDE5 inhibitor or Sch51866 as a selective PDE9 inhibitor. In the presence of PDE inhibition, cGMP content increased markedly after stimulation of the particulate guanylyl cyclase. mRNA of PDE2, PDE5 and PDE9 was detected in all cultured human RPE cells and also in rat RPE cell layers.

Conclusions: PDE2, PDE5 and PDE9 have a role in cGMP metabolism in RPE cells.

Individual cells were grouped in defined grey value classes. Numbers are the means of three experiments (six pictures were taken per incubation in each experiment) and they express the percentage of cells in each of the four intensity classes.

Cyclic 3',5'-guanosine monophosphate (cGMP), a central molecule in the phototransduction cascade,^{1,2} is also involved in several other physiological processes in the retina. cGMP stimulates the absorption of subretinal fluid by activating the retinal pigment epithelium (RPE) cell pump.^{3,4} Recently, we found that retinal detachment was associated with a decrease in the cGMP concentration in the subretinal fluid as compared with controls.⁵ These findings prompted us to investigate the mechanisms involved in retinal cGMP metabolism.

Intracellular concentrations of cGMP represent the net balance between synthesis by guanylyl cyclases and breakdown by 3',5' cyclic nucleotide phosphodiesterases (PDEs).⁶ PDEs comprise a large group of enzymes that hydrolyse cyclic adenosine monophosphate (cAMP) and cGMP to their inactive 5' derivatives. Eleven different subfamilies of PDE isozymes (PDE1–PDE11) are known. The classification is based on their affinities for cGMP and cAMP, and several proteomic and genomic characteristics.⁷ PDE2, PDE5, PDE 6, PDE 9 and PDE10 are the major cGMP-degrading enzymes.^{8–12} PDE10, which hydrolyses both cAMP and cGMP, was however not detected in the eye, and PDE6 is known to be present only in retinal photoreceptor cells.^{13,14}

cAMP-hydrolysing and cGMP-hydrolysing activities have been detected in homogenates of cultured pigment epithelium from rats.¹⁵ Although the first papers about PDE activity in RPE cells were already published in the early 1980s,^{16,17} no data are available on the expression of the individual PDE isoforms that have been characterised over the past 10 years.

RPE cells are in close contact with the photoreceptor layer and facilitate the supply of necessary substrates, including oxygen, to the photoreceptor cells. We have found that RPE cells are capable of producing cGMP and most cGMP is

generated after stimulation of the particulate guanylyl cyclase pathway and simultaneous non-selective PDE inhibition with 3-isobutyl-1-methylxanthine (IBMX; Diederer, unpublished data).

The aim of this study was to identify the PDE isoforms involved in the cGMP breakdown in RPE cells. To investigate PDE activity in RPE cells, we studied cGMP accumulation in the RPE cells after stimulating particulate or soluble guanylyl cyclase in the presence of PDE inhibitors with different selection properties. We used IBMX as a non-specific PDE inhibitor, Bay 60–7550 as a selective PDE2 inhibitor,¹⁸ sildenafil as a selective PDE5 inhibitor and Sch 51866 as a selective PDE9 inhibitor.¹⁹ In addition, mRNA expression of PDE2, PDE5 and PDE9 was studied in cultured human RPE cells using non-radioactive *in situ* hybridisation. As it is impossible to detect the *in situ* hybridisation signal in pigmented RPE cells, we studied the *in vivo* situation by analysing the mRNA expression of PDE2, PDE5 and PDE9 in the unpigmented RPE cell layer of albino Lewis rats.

Our results indicate that PDE2, PDE5 and PDE9 are present in cultured human RPE cells and rat RPE cell layers, and that these different PDE isoforms have a role in controlling cGMP levels in RPE cells.

METHODS

Cell culture

Human RPE cells (D407 cell line)¹⁹ were suspended in Dulbecco's modified Eagle's medium containing 5% fetal calf serum, 2 mM glutamine and 100 µg/ml penicillin streptomycin, at a concentration of 0.5–1.0 × 10⁶ viable cells/ml. Portions of the suspension (1 ml) were seeded on to 40 mm diameter

Abbreviations: ANP, atrial natriuretic peptide; cAMP, cyclic adenosine monophosphate; cGMP, cyclic 3',5' guanosine monophosphate; DIG, digoxigenin; IBMX, 3-isobutyl-1-methylxanthine; PDE, phosphodiesterase; RPE, retinal pigment epithelium; SNP, sodium nitroprusside; SSC, TBS, TRIS-buffered saline; TBS-T, TRIS-buffered saline Triton X

Table 1 cyclic 3'5' guanosine monophosphate fluorescence intensity in human retinal pigment epithelium cells after incubation with different phosphodiesterase inhibitors and guanylyl cyclase stimulation

Treatment	n	300–600	Grey Values			
			600–1500	1500–2700	2700–4095	
Control	3	95	5	0	0	
+SNP	3	87	13	0	0	
+ANP	3	95	5	0	0	
IBMX	3	65	24	11	0	
+SNP	3	64	34	2	0	
+ANP*	3	22	39	32	7	
Sildenafil	3	91	9	0	0	
+SNP	3	96	4	0	0	
+ANP*	3	18	60	10	12	
BAY 60–7550	3	99	1	0	0	
+SNP	3	86	14	0	0	
+ANP*	3	01	88	5	6	
Sch51866	3	99	1	0	0	
+SNP	3	93	7	0	0	
+ANP*	3	40	46	14	0	

ANP, atrial natriuretic peptide; IBMX, 3-isobutyl-1-methylxanthine; SNP, sodium nitroprusside

$p < 0.001$ when compared with control +ANP.

plastic petridishes and incubated at 37°C in a humidified atmosphere of 95% air and 5% CO₂.

Pharmacology

Twenty four hours after seeding, the subconfluent RPE cells were incubated for 40 min in aerated Krebs buffer (pH 7.4) with or without the following compounds: 1 mM IBMX (non-specific PDE inhibitor; Sigma), 1.0 μM Bay 60–7550 (selective PDE2 inhibitor),¹⁹ 10 μM sildenafil (selective PDE5 inhibitor) and 10 μM Sch51866 (selective PDE9 inhibitor).¹³ During the last 10 min of the incubation, the cells were stimulated with 100 μM of the NO donor sodium nitroprusside (SNP) or 0.1 μM atrial natriuretic peptide (ANP).

Immunohistochemistry

After fixation with 4% freshly depolymerised paraformaldehyde in the cold (4°C), the cells were washed 3×5 min, twice with TRIS-buffered saline (TBS) and once with TBS containing 0.3% Triton X-100 (TBS-T). Cells were incubated overnight at 4°C

with the primary antibodies. cGMP was visualised using sheep polyclonal anti-formaldehyde-fixed cGMP antiserum diluted 1:4000 in TBS-T.²⁰ Cells were washed for 15 min with TBS, TBS-T and TBS, respectively. The cells were then incubated with Alexa 488 conjugated donkey anti-sheep immunoglobulin (Ig)G (Molecular Probes) diluted 1:100 in TBS-T for 1.5 h at room temperature. Negative controls were processed in exactly the same way, with the omission of the primary antibody. After three further washings with TBS, the cells were cover-slipped with TBS/glycerol (1:2 v/v).

Rats

Adult male Lewis rats (200–240 g) were reared under standard conditions and were cared for in accordance with the guidelines of the Association for Research in Vision and Ophthalmology Statement for the use of animals in ophthalmic and vision research. The animals were decapitated and their eyes were immediately frozen. Frozen sections of 10 μm were cut and kept at –80°C until the experiment was completed.

In situ hybridisation of PDE2, PDE5 and PDE9

In situ hybridisation was performed with digoxigenin (DIG) labelled RNA probes as described previously.²¹ Frozen sections were thawed for 10 min at 50°C and thereafter fixed with 4% paraformaldehyde in 0.1 M phosphate buffer for 20 min at room temperature. The RPE cells were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer for 30 min at 4°C. The samples (sections and cells) were then washed shortly with phosphate buffered saline and incubated for 10 min with 0.1 M triethanolamine containing 0.25% acetic anhydride. Samples were washed two times with 2× SSC and thereafter washed at 37°C with 2× SSC containing 50% formamide. Hybridisation was performed overnight in a humid chamber at 55°C under cover slips in 100–200 μl hybridisation mix (50% (v/v) deionised formamide, 250 μg/ml salmon sperm DNA, 1 mg/ml tRNA, 10% dextran sulphate, 2× SSC, 1× Denhardt's solution and 200 ng/ml DIG-labelled RNA probe). After hybridisation, the samples were washed in 2× SSC, 1× SSC and 0.1× SSC: all solutions containing 50% formamide. Each wash step was performed at 55°C for 20 min. Next, the samples were treated with RNase T1 2 U/ml (Roche) in 2× SSC containing 1 mM EDTA for 15 min at 37°C, followed by a 20 min wash with 1× SSC and a 10 min wash with 2× SSC. The samples were then incubated for 5 min with buffer 1

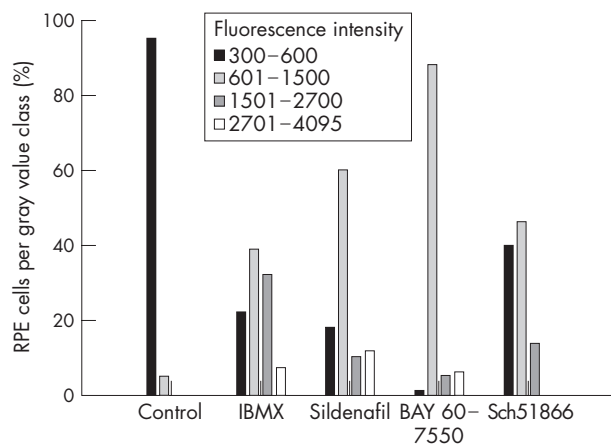


Figure 1 Cyclic 3',5' guanosine monophosphate fluorescence intensity in human retinal pigment epithelium cells after incubation with different phosphodiesterase inhibitors and guanylyl cyclase stimulation with 0.1 μM atrial natriuretic peptide. Individual cells were grouped in defined grey value classes. Numbers are the means of three experiments (six pictures were taken per incubation in each experiment) and they express the percentage of cells in each of the four intensity classes.

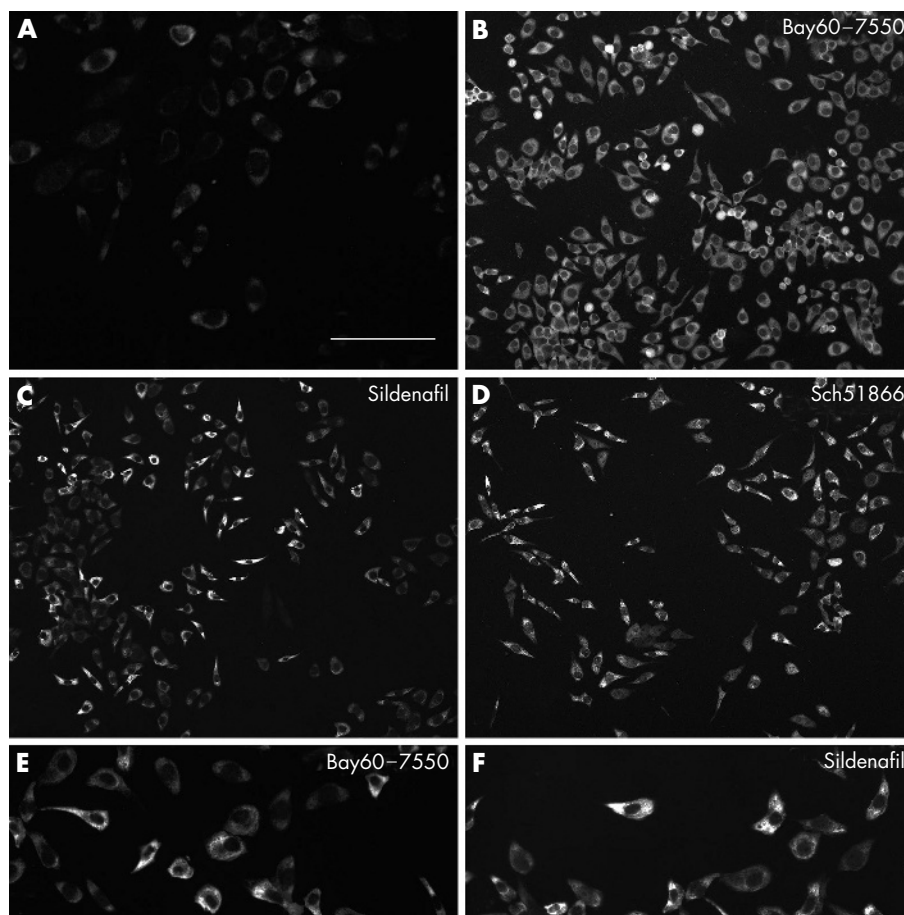


Figure 2 Cyclic 3',5'-guanosine monophosphate immunoreactivity in human retinal pigment epithelium cell. (A) Without phosphodiesterase inhibition. (B, E) Combination of 1.0 μ M Bay 60-7550 and 0.1 μ M atrial natriuretic peptide. (C, F) Combination of 10 μ M sildenafil and 0.1 μ M ANP. (D) Combination of 10 μ M Sch51866 and 0.1 μ M ANP. Sections incubated without primary antibody show no cGMP immunoreactivity (not shown). Pictures are not taken from the same experiment. Scale bar is 100 μ m in B-D 50 μ m in A, E and F.

(0.15 M NaCl and 0.1 M maleic acid (pH 7.5)) followed by blocking for 2 hr at reverse transcription with buffer 2 (0.15 M NaCl, 0.1 M maleic acid (pH 7.5) and 1% blocking reagent (Roche, 1096176)) containing 5% sheep serum. Next, samples were incubated overnight with a 1:2000 dilution of anti-DIG-alkaline phosphatase (Roche) in buffer 2 containing 1% sheep serum. Samples were washed three times with buffer 1, followed by washing for 10 min with TBS containing 0.025% Tween 20 and thereafter, three times for 5 min with TBS. After two washes in buffer 3 (0.1 M TRIS-HCl (pH 9.5), 0.1 M NaCl and 0.05 M magnesium chloride), the samples were incubated with freshly prepared nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate in buffer 4 (0.05 M TRIS (pH 9.5) 0.1 M NaCl, 6% polyvinylalcohol, 0.05 M $MgCl_2$, 1 mM levamisole) and stained overnight in the dark at 25°C. The colour reaction was stopped with 0.01 M TRIS-HCl-1 mM EDTA (pH 8.0).

Quantification of immunostainings

Immunostainings were examined using an Olympus A \times 70 microscope equipped with a cooled charge couple devices Olympus F-view camera. Experiments were repeated three times. From each incubation setting, six images were randomly chosen. Each image contained at least 50 RPE cells. Fluorescence intensities were converted into grey values (AnalySIS, Softimaging system) ranging from 0 (blank) to 4096 (white). After establishing a threshold setting using a

blank preparation incubated with the secondary antibody only, five classes with increasing grey values were set as 0-300 (blank), 301-600, 601-1500, 1501-2700 and 2701-4096. All cells were classified according to the highest grey value they showed (table 1). The mean percentage of cells in each class was calculated for the six images taken from each incubation setting. The mean percentage was calculated for the three experiments. The distributions of the cells over the different classes were analysed using the χ^2 test.

RESULTS

Effect of PDE inhibition on cGMP levels in cultured human RPE cells

In unstimulated cells incubated without PDE inhibition, a low level of cGMP immunoreactivity was visualised in the cells (table 1; fig 2A). Hardly any differentiation in the intensity of the cGMP immunofluorescence was present. Addition of 1 mM IBMX to the incubation medium resulted in a general increase in the intensity of the cGMP immunostaining. We also observed more differentiation in the intensity of the cGMP immunofluorescence seen compared with the intensity after incubation without PDE inhibition (table 1).

Incubation of the cells without stimulation of SGC or pGC, although in the presence of Bay 60-7550 (PDE2 inhibitor), sildenafil (PDE5 inhibitor) and Sch51866 (PDE9 inhibitor), did not increase cGMP immunoreactivity compared with incubation without PDE inhibition (table 1).

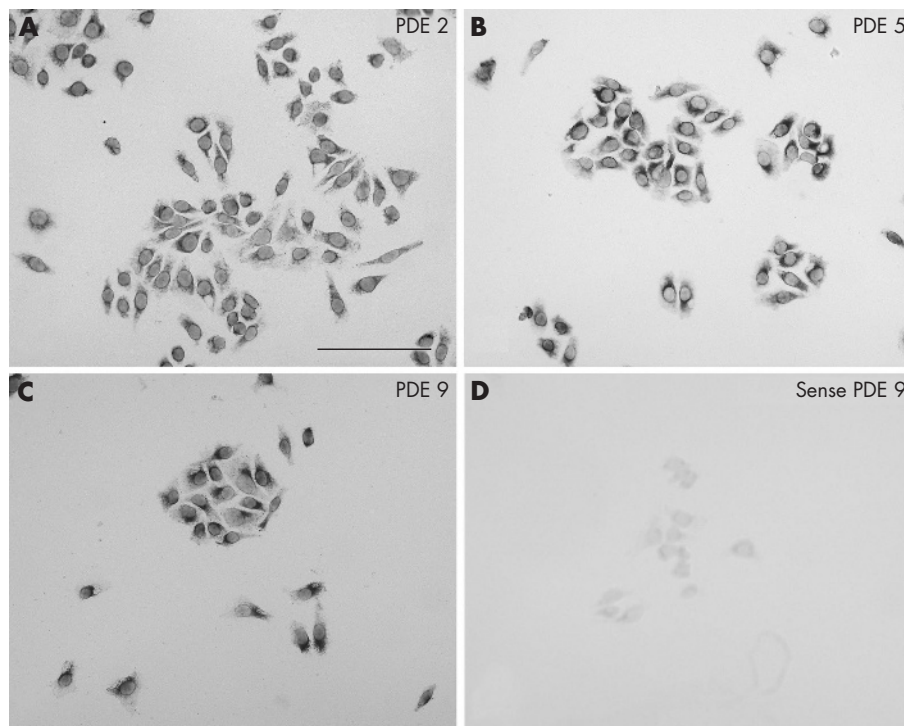


Figure 3 In situ hybridisation of phosphodiesterase PDE2, PDE5 and PDE9 in human retinal pigment epithelium cells. Localisation of (A) PDE2 mRNA, (B) PDE5 mRNA and (C) PDE9 mRNA. (D) Hybridisation with the sense probe of PDE9. Scale bar is 100 μ m.

Exposure of RPE cells to 0.1 μ M ANP in combination with Bay60-7550, sildenafil or Sch51866 resulted in a strong increase in cGMP immunoreactivity in a large number of cells (figs 1 and 2). Figures 1 and 2 show that the effect of sildenafil

and IBMX are quite similar, both with several cells in the highest grey value intensity class. The effect of Bay60-7550 is more homogenous in all cells, resulting in 88% of the cells in the second grey value intensity class. The distribution of GMP

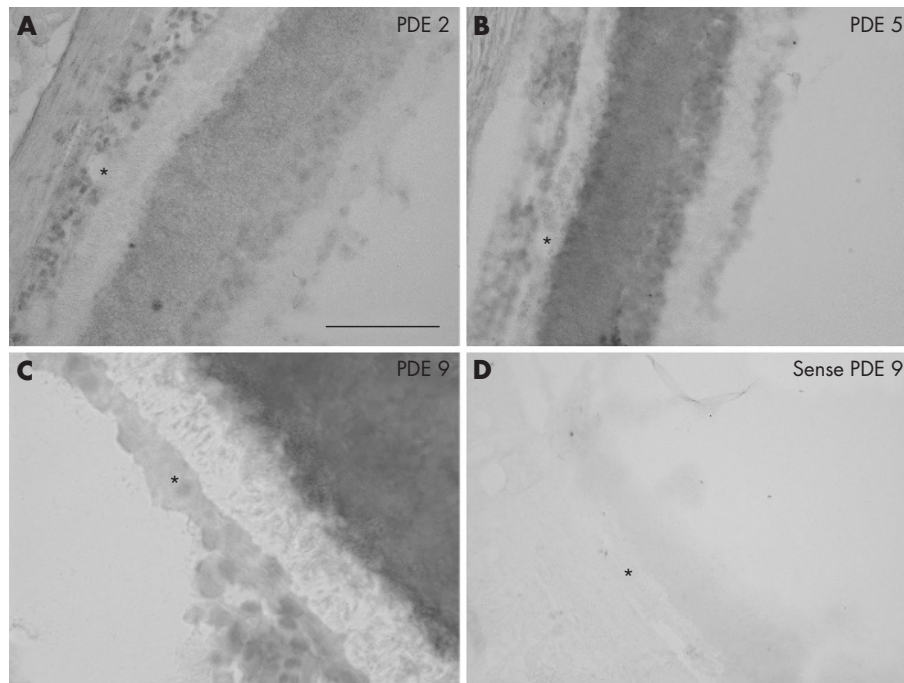


Figure 4 In situ hybridisation of phosphodiesterase PDE2, PDE5 and PDE9 rat retinal pigment epithelium cell layers. Localisation of (A) PDE2 mRNA, (B) PDE5 mRNA and (C) PDE9 mRNA (D) Hybridisation with the sense probe of PDE9. *RPE cell layer. Scale bar is 100 μ m in A, B and D. Scale bar is 50 μ m in C.

throughout the cytoplasm is not homogeneous but seemed to be compartmentalised in many cells (fig 2). This holds true for all three PDE inhibitors. These results show that Bay 60–7550 sildenafil and Sch51866 were able to inhibit PDE activity. We found no major stimulating effect of SNP on cGMP synthesis in the presence of all three PDE inhibitors.

mRNA expression

Non-radioactive in situ hybridisation was used to study the mRNA expression patterns of PDE2, PDE5 and PDE9. RPE cells were hybridised with antisense and sense probes from these three different PDE families. In human RPE cell cultures, PDE 2, PDE5 and PDE9 mRNAs were detected in all cells, and most of the mRNA was visualised concentrated around the nucleus (fig 3). Control sections hybridised with sense probes did not show any specific staining.

Rat RPE cell layers were also hybridised with antisense and sense probes from PDE2, PDE5 and PDE9, and mRNA of PDE 2, PDE5 and PDE9 was shown to be present (fig 4). The in situ hybridisation signal was present in all RPE cells almost to the same extent. Control sections hybridised with sense probes did not show any specific staining.

DISCUSSION

We investigated the contribution of three isoforms of the PDE family of enzymes in controlling cGMP levels in human RPE cell cultures. Data obtained by using selective PDE inhibitors in combination with cGMP immunocytochemistry indicate that at least PDE 2, PDE5 and PDE9 are present in cultured human RPE cells. This was confirmed by the presence of mRNA of PDE 2, PDE5 and PDE9 in the human RPE cells and rat RPE cell layers studied by using in situ hybridisation. These three different PDE isoforms seem to be involved in controlling cGMP levels in RPE cells, as PDE10 was found not to be present in the eye.¹³ Differences in mRNA expression between cells may be caused by cells being in a different cell cycle phase. Therefore, it is not surprising that a similar difference in the localisation of cGMP immunocytochemistry was observed in the cells, irrespective of the PDE inhibitor being present during the incubation.

PDE2 has an almost equal affinity for cGMP and cAMP. However, cAMP hydrolysis is greatly stimulated by low levels of cGMP.¹⁴ The compound Bay60–7550 is a highly selective inhibitor of PDE2 and more potent than erythro-9-(2-hydroxy-3-nonyl) adenine, which is generally used as a PDE2 inhibitor.¹⁸ In addition, in contrast to erythro-9-(2-hydroxy-3-nonyl) adenine, Bay 60–7550 is devoid of adenosine deaminase activity.¹⁸

PDE5 has been implicated in the control of vascular resistance.¹⁴ Also in the retina, PDE5 was shown to have a possible role in the regulation of retinal blood flow.²³ Sildenafil, a relatively selective inhibitor for PDE5, also weakly inhibits PDE6, an enzyme involved in the process of phototransduction, with an efficacy of 1/10 of that for PDE5.²³ A single oral dose of 100 mg sildenafil causes impaired photoreceptor function, which is being attributed to weak inhibition of PDE6 by sildenafil.^{24–25} To date, PDE6 is known to be present only in retinal photoreceptor cells.¹⁴

As PDE9 has the highest affinity for cGMP of all the mammalian PDEs, this enzyme can have a fundamental role in keeping cGMP at a low basal level.⁹ In contrast with the other PDEs, PDE9 is not inhibited by IBMX.^{11–13} Sch51866 is a somewhat selective inhibitor of PDE9 as it also weakly inhibits PDE5, with an efficacy of 1/15 of that for PDE9.¹⁹ So the effect of Sch51866 on the cGMP immunoreactivity in the current study cannot be solely attributed to inhibition of PDE9.

Using in situ hybridisation, we showed the presence of PDE2, PDE5 and PDE9 mRNA in RPE cells, but this is not a quantitative technique. The immunohistochemistry data clearly show that a combination of sildenafil and ANP results in the largest percentage of cells in the highest grey value intensity class compared with other incubation settings. This may indicate a prominent role of PDE5 in cGMP breakdown in RPE cells.

The D407 cell line was shown to retain many of the metabolic and morphological characteristics of RPE cells in vivo, although there are some minor differences.¹⁸ Given our results, it may be concluded that the expressions of PDE2, PDE5 and PDE9 are similar in the D407 cell line compared with those in rat RPE cells in vivo.

The major function for PDEs in the cell is to terminate the amplitude and duration of the cyclic nucleotide second messenger signal.¹⁴ As shown previously, retinal detachment is associated with a decrease in the cGMP concentration in the subretinal fluid.⁵ Hypoxia due to retinal detachment may cause a reduced activity of guanylyl cyclases that produce cGMP. This could lead to a reduced expression of cGMP by retinal cells after retinal detachment. By knowing the specific PDE families located in retinal cells, these enzymes could in the future become therapeutic targets to limit cGMP breakdown by using selective PDE inhibitors. Clinically, this could be important as cGMP is known for its stimulating role in the reabsorption of the subretinal fluid by activating the RPE pump.^{3–4}

In conclusion, our results show that there is a complex regulation of cGMP synthesis in RPE cells. The three PDE isoforms, PDE2, PDE5 and PDE9, are involved in the breakdown of cGMP in these cells. This process of controlling cGMP levels might have a role in the regulation of fluid absorption from the subretinal space by RPE cells.

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