Figure 2. In vitro expression of cyclic guanosine monophosphate (cGMP) in the photoreceptor layer of a normal or detached pig retina. Small pieces of retina were incubated with IBMX (a non-specific PDE inhibitor) and the particulate guanylyl cyclase (pGC) stimulator atrial natriuretic peptide (ANP). The expression of cGMP (cGMP: green color) in the retina was then analyzed by immunohistochemistry and nuclei were counterstained with Hoechst 33342 (red). Under these conditions no cGMP signal was visible in the photoreceptor layer of the healthy retina (Panel A). The detached retina on the other hand displayed strong cGMP expression in the photoreceptor layer (Panels B and C). Panel B is a double staining exposure for the nuclei and cGMP, showing the presence of cGMP in the nuclei of the cells (yellow) and in the cytoplasma (green). In Panel C we only show the green signal, showing strong cGMP immunoreactivity in the same piece of retina. Scale bar represents 25 μm.

the incubation, the tissue was stimulated with 100 μM of the NO donor SNP or 0.1 μM of the pGC stimulator ANP.

Immunohistochemistry: To determine the site of cGMP production in the pig retina the following experiments were performed. As mentioned in the previous paragraph, pieces of retinal tissue from eyes with and without a retinal detachment were incubated in vitro, and cGMP production was induced by stimulation of the sGCs (by SNP) or pGCs (by ANP) with a simultaneous inhibition of cGMP breakdown by PDEs using IBMX. After incubation the retinal tissue pieces were fixed with 4% freshly depolymerized cold (4 °C) paraformaldehyde. Retina wholemounts were washed 3 times for 5 min, twice with Tris-buffered saline (TBS) and once with TBS containing 0.3% Triton X-100 (TBS-T). Samples were subsequently incubated overnight at 4 °C with the primary antibodies. Cyclic GMP was visualized using sheep antiformaldehyde-fixed cGMP antiserum diluted 1:4000 in TBS-T as we described earlier [18].

The following day, the samples were washed for 15 min with TBS first, then TBS-T and TBS. The tissues were subsequently incubated with Alexa 488 conjugated donkey antisheep IgG (Molecular Probes, Breda, The Netherlands), 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. The tissues were washed three more washings in TBS, and the nuclei of the cells were stained with Hoechst 33342, (Sigma, Zwijndrecht, The Netherlands) for 20 min at room temperature (diluted 1:1000) and finally washed again in TBS. Wholemounts were coverslipped with TBS:glycerol (1:2 v/v).

Microscopy: Retinal wholemounts were analyzed by fluorescence microscopy using two-photon laser scanning microscopy (TPLSM). TPLSM was performed as previously described using a microscope objective (40x; water dipping; numerical aperture [NA]=1.0) connected to an upright Nikon E600FN microscope (Nikon Corporation, Tokyo, Japan) [19]. Further magnification, when needed, was achieved by an optical zoom in the scan head. To remove background noise, we filtered each image using the Kalman filtering procedure on three subsequent images during experiments. The fluorescent secondary antibody Alexa 488 was mainly visible in the green channel, whereas Hoechst was only visible in the red channel. The obtained images (coded green and red, respectively) were combined into single images as needed. Images were taken with a MBF Bioscience Stereo Investigator Confocal Spinning Disk (SI-SD) system (MBF Bioscience, Williston, VT), consisting of a modified Olympus BX51 fluorescence microscope (Olympus, Tokyo, Japan) with UPlanSApo objectives, customized spinning disk unit (DSU; Olympus, Zoeterwoude, The Netherlands), computer-controlled excitation and emission filter wheels (Olympus), three-axis high-accuracy computer-controlled stepping motor specimen stage (4x4 Grid Encoded Stage; Ludl Electronic Products, Hawthorne, NY), linear z-axis position encoder (Ludl), ultra-high sensitivity monochrome electron multiplier CCD camera (1,000x1,000 pixels, C9100–02; Hamamatsu Photonics, Hamamatsu City, Japan) and controlling software (MBF Bioscience). Pictures were taken in a single focal plane with a 40x UPlanSApo objective (N.A.=0.9) and processed with Imaris software (Version 4.0; Bitplane, Zurich, Switzerland). Only minor adjustments of contrast and brightness were made without altering the appearance of the original materials. No deconvolution was performed on the images. The computer-based stereology system that was used
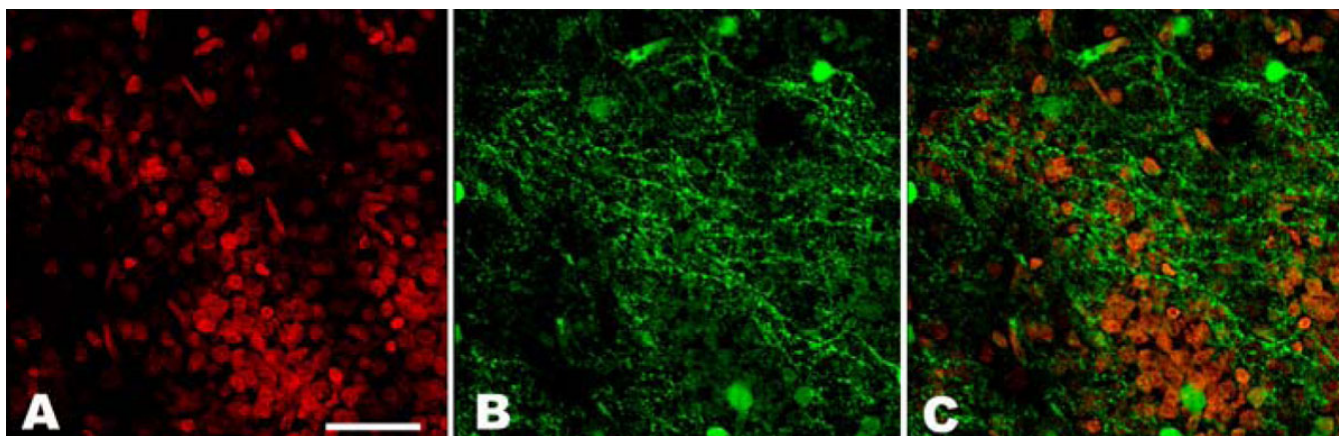
Figure 3. In vitro expression of cyclic guanosine monophosphate (cGMP) in the outer plexiform layer of a detached retina. Small pieces of retina were incubated with IBMX (a non-specific PDE inhibitor) and the particulate guanylyl cyclase (pGC) stimulator atrial natriuretic peptide (ANP). The expression of cGMP (cGMP: green color) in the retina was then analyzed by immunohistochemistry and nuclei were counterstained with Hoechst 33342 (red). Following stimulation with ANP in the presence of IBMX, both the detached and healthy retina showed cGMP-immunolabeling in the outer plexiform layer. Panel A shows the labeling of the outer plexiform layer of a detached retina with Hoechst. Panel B shows the cGMP stain (green) of the outer plexiform layer probably representing photoreceptor axons and dendrites. Panel C is a double staining exposure of the nuclei and cGMP. Scale bar represents 20 μm.

allows encoding of the Z-axis position thereby measuring the actual focus position within the retina.

Statistical analysis: Because the cGMP values were paired and nonparametric, we used the Wilcoxon signed ranks test to compare the cGMP content in pig vitreous fluid of eyes with and without retinal detachment. Differences were considered significant when the p value was less than 0.05 (two-tailed).

RESULTS

We were able to detect cGMP in all the vitreous fluid samples obtained from the pig eyes used in this study. The mean vitreous level of cGMP was 1.45 pmol/ml (range: 0.09 to 4.32 pmol/ml) in eyes with retinal detachment. In the control (contralateral) eyes, the mean vitreous level of cGMP was 4.61 pmol/ml (range between 0.40 to 14.10 pmol/ml). The difference in vitreous cGMP levels between eyes with detached retinas and control eyes was significant (p=0.028 by Wilcoxon signed ranks test). A large variation in cGMP levels was observed between pigs. In each pig, however, the cGMP level in the right eye with a retinal detachment was always lower (between 1.6 and 5.8 times) than in the healthy left eye (Figure 1).

Immunohistochemistry: The expression of cGMP in the retina was analyzed by immunohistochemistry. No cGMP expression could be observed in any of the retinal cells (either detached or healthy) if phosphodieasterase activity had not been blocked with IBMX. This indicates that under normal conditions, cGMP has an extremely short half life. In the absence of cyclase stimulation but in the presence of PDE blockers, we observed cGMP immunoreactivity in the inner nuclear layer of the detached as well as the healthy retinas. Addition of 0.1 μM ANP as a stimulator of pGC, combined with PDE inhibition, resulted in the appearance of a strong

cGMP signal in both the ganglion cells and the inner nuclear layer of the healthy retina. No cGMP signal was visible in the outer nuclear layer of the healthy retina (Figure 2A). The detached retina on the other hand displayed strong cGMP expression not only in the ganglion cells and the inner nuclear layer but also in the outer nuclear layer (Figure 2B,C). Figure 2B shows both the nuclei of the cells (Hoechst staining; red signal) and cGMP (green signal) staining of the outer nuclear layer of a detached retina. Figure 2C only presents the green signal, showing strong cGMP immunoreactivity in the same piece of retina. Following stimulation with ANP in the presence of IBMX, both the control eye and eye with the detached retina showed cGMP-immunolabeling in the inner and outer plexiform layer. Figure 3 presents the labeling of the outer plexiform layer of a detached retina with Hoechst (Figure 3A) and cGMP (Figure 3B) separately and together (Figure 3C) in the same piece of retina. Incubating the retinas in the presence of 100 μM SNP, as a stimulator of sGC in combination with PDE inhibition (with IBMX) resulted in cGMP-immunoreactivity in the ganglion cells, the inner nuclear cell layer (Figure 4 C,D) and the nerve fiber layer of the detached- and healthy retinas (Figure 4A,B). Stimulation with SNP (+ IBMX) did not result in the appearance of cGMP immunoreactivity in the photoreceptor cells of the detached or healthy retina. Incubation of retinas with ANP or SNP (+ IBMX) both resulted in cGMP-immunoreactivity in the inner nuclear layer, although not in all cell types (Figure 4C,D). The aforedescribed experiments thus only showed a difference in cGMP expression between the retinas with and without experimental detachment following in vitro stimulation of pGC. In vitro, we demonstrated that ANP induced synthesis of cGMP in the outer nuclear layer of the detached retina, whereas this was not the case in the attached retina.
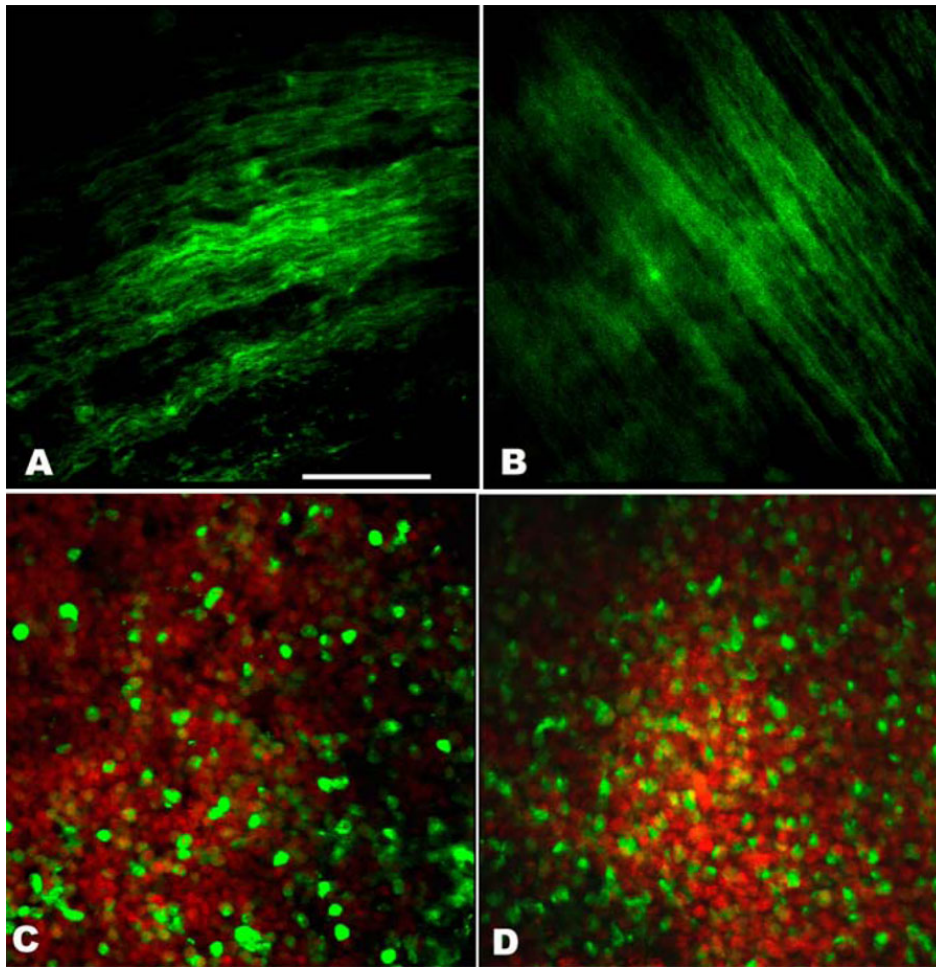
Figure 4. In vitro expression of c cyclic guanosine monophosphate (cGMP) in the nerve fiber layer and inner nuclear layer of a healthy or detached retina. Small pieces of retina were incubated with IBMX (a non-specific PDE inhibitor) and the soluble guanylyl cyclase (sGC) stimulator sodium nitroprusside (SNP). The expression of cyclic GMP (cGMP: green color) in the retina was analyzed by immunohistochemistry and nuclei were counterstained with Hoechst 33342 (red). Panels A and B show a similar cGMP expression in nerve fiber layer of the normal (A) or detached (B) retina. Panel C (normal retina) and Panel D (detached retina) show cGMP staining in the inner nuclear cells stained in red. Scale bar represents 50 μm.

DISCUSSION

In the current study, we demonstrated the presence of cGMP in pig vitreous fluid and showed a significantly lower vitreous cGMP level following retinal detachment. The animal model thus confirms earlier findings in humans in which we showed a significantly lower vitreous cGMP concentration in patients with an rhegmatogenous retinal detachment as compared to controls [6]. Our findings are also in accordance with earlier findings in a rabbit model that showed that vitreous cGMP levels were almost 60% lower in eyes with a retinal detachment than in control eyes [20].

Cyclic GMP production is catalyzed by either sGC, activated by NO or pGC stimulated by natriuretic peptides. After retinal detachment, the activity of both sGC and pGC could be reduced, since certain populations of retinal cells, such as the photoreceptor or Müller cells, may have undergone degenerative changes induced by hypoxic or ischemic alterations following the detachment [4,5]. A decreased production of cGMP by retinal cells may concurrently lead to a lower release into the extracellular space--in this case, vitreous fluid. This explanation is speculative, however, since no evidence is available to date concerning extracellular

transport of cGMP from retinal cells. Previously we showed that in vitro cultured RPE cells can be triggered to produce and secrete cGMP into the culture medium [21]. A similar approach could be used with retinal sections from healthy and detached retinas to assess a possible contribution of sensory retinal cells to the level of cGMP in vitreous or subretinal fluids.

We measured cGMP in vitreous fluid and retinal sections using immunoassays. The presence of cGMP immunoreactivity is considered to be equivalent to a potential biologic activity of the cGMP molecule as we recently showed in a model of rat aorta contraction and dilation [22]. Biologic activity of cGMP depends on its targets, which include the cGMP-dependent protein kinases, cyclic nucleotide-gated channels, cAMP-dependent protein kinase, and PDEs [23]. The actual presence of these targets may differ among cells, explaining the wide variety of activities of cGMP.

The cellular concentration of cGMP represents the net balance between synthesis by guanylyl cyclases and breakdown into 5′-cGMP by cyclic nucleotide PDEs [24]. The decreased cGMP concentration in the vitreous after retinal detachment could also be the result of increased PDE activity.

This seems less plausible, however, since no detectable PDE activity seems to be present in vitreous fluid [6]. After rhegmatogenous retinal detachment, vitreous passes through a retinal break into the subretinal space and then comes into direct contact with the RPE layer. It is possible that the cGMP in the vitreous is degraded by PDEs located in the RPE cells. This may be plausible since earlier studies have shown the presence of at least PDE 2, PDE 5, and PDE 9 in RPE cells [25].

Stimulation of sGC by the NO donor SNP resulted in cGMP expression in the inner pig retina, especially in the ganglion cells and bipolar cell layer. Further studies are needed to determine the pathways that are targeted by cGMP within these cells.

The effect of SNP on cGMP immunoreactivity in the pig retina was similar to findings reported in other species [9, 10]. After stimulation of the particulate guanylyl cyclase with ANP, cGMP expression was observed in the outer nuclear layer of the eyes with a detached retina. Under similar conditions the outer nuclear layer of the eyes with a normal attached retina did not express cGMP. The explanation for this difference remains speculative. It may be caused by changes in local PDE levels or by changes in the ANP receptor expression of cells in the outer retina. Further analysis on retinal PDE expression following detachment is necessary to fully understand the dynamics of cGMP in the retina.

The increased cGMP expression in the detached retina compared with the normal retina seems to contradict the finding that cGMP levels were lower in the vitreous of eyes with a detached retina. On the other hand this could also be explained by assuming a decreased secretion of cGMP by the retinal cells leading to lower levels in the extracellular space and concomitant intracellular accumulation.

Most studies concerning retinal cGMP have focused on its role in phototransduction [26]. Little is known about the changes in cGMP expression in the retina and the vitreous after retinal detachment. It has been shown that cGMP stimulates subretinal fluid absorption whereas cAMP inhibits this process [27]. Until now, we have only measured cGMP in ocular fluids following clinical or experimental detachments. Simultaneous determination of cAMP may be useful for the understanding of subretinal fluid dynamics after the formation of retinal break.

ACKNOWLEDGEMENTS

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