Natriuretic Peptide Receptors, NPR-A and NPR-B, in Cultured Rabbit Retinal Pigment Epithelium Cells

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ABSTRACT—We tried to detect natriuretic peptide (NP) receptor (NPR-A and NPR-B) mRNAs in cultured rabbit retinal pigment epithelium (RPE) cells and examined the regulation of their expression in relation to subretinal fluid absorption or RPE cell proliferation. RPE cells from 2–4 passages were grown to confluence on microporous membranes and analyzed for levels of expression of receptor mRNAs by quantitative RT-PCR and Northern blotting. The expression of NPR-B mRNA was approximately tenfold higher than that of NPR-A mRNA. The expression of NPR-A mRNA was not affected by treatments that may change subretinal fluid transport, while that of NPR-B mRNA was inhibited by transmitters involved in light- and dark-adaptation such as dopamine and melatonin. Expression of NPR-B mRNA was also suppressed by platelet-derived growth factor and transforming growth factor-β. Furthermore, atrial natriuretic peptide (ANP) and C-type natriuretic peptide (CNP), ligands for NPR-A and B, respectively, inhibited the proliferation of RPE cells, as analyzed by incorporation of [3H]thymidine. These findings suggest that ANP may be involved in constitutive absorption of subretinal fluid and that NPs form an important regulatory system of proliferation in RPE cells.

Keywords: Retinal pigment epithelium (RPE), Atrial natriuretic peptide (ANP), C-type natriuretic peptide (CNP), Natriuretic peptide receptor-A (NPR-A), Natriuretic peptide receptor-B (NPR-B)

The retinal pigment epithelium (RPE) is a single cell layer that forms a diffusion barrier at the back of the eye and regulates the transport of fluid, ions and metabolites between the neural retina and its choroidal blood supply.

Among the natriuretic peptides (NPs), atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) are thought to affect the regulation of extracellular fluid volume and blood pressure (1). They are produced predominantly by the heart and to less extents by extracardiac tissues and act not only as circulating peptides but also as locally synthesized molecules (1). In contrast, a third peptide, C-type natriuretic peptide (CNP), has been found mainly in the central nervous system (1, 2) and endothelial cells (3), and this peptide acts as a local vascular regulator with little natriuretic activity (4). Most of the biological actions of NP are thought to be mediated by two guanylate cyclase-linked receptor subtypes (NPR-A and NPR-B) with different ligand selectivities (5). NPR-A is activated by ANP and BNP, whereas CNP is considered to be the specific ligand of NPR-B. All three NPs bind to the C-type receptor (NPR-C). This type of receptor lacks guanylate cyclase activity and has clearance function for NPs (6).

In the eye, specific high-affinity binding sites and binding capacities for [125I]-ANP and/or [125I]-BNP have been identified in the rat ciliary process and retina (7, 8) and in human retina and RPE (9). Recently, NPR-A and NPR-B mRNAs have been detected in the rat ciliary process, retina and choroid (10–12). The transcripts encoding ANP, BNP and CNP were also detected in the ciliary process and choroid, whereas only BNP and CNP mRNAs were observed in retina (12, 13). These findings, together with the observations that ANP can decrease intraocular pressure (14, 15), suggest that the NPs may regulate the transport of ocular fluid via autocrine or paracrine mechanisms. However, the effects of the NP on the subretinal fluid transport mediated by RPE cells have not been well characterized, and the function of CNP in
the eye as well as the existence of NPR-A and NPR-B mRNAs in RPE cells have not been shown.

We showed recently that in RPE cells, ANP facilitated Cl⁻ transport mediated by the Na⁺/K⁺/2Cl⁻ cotransporter (16), which is known to play a major role in driving subretinal fluid (17). In recent years, the NPs have been demonstrated to inhibit the proliferation of a variety of cells (18–20), and it has been shown that the proliferation of RPE cells profoundly affects some kinds of pathogenesis of the eye. In the present study, we detected NPR-A and NPR-B mRNAs in rabbit cultured RPE cells and evaluated alterations of their expression by treatments that may change the subretinal fluid transport or the proliferation of RPE cells. In a second set of experiments, we examined the effects of ANP and CNP on the proliferation of RPE cells.

MATERIALS AND METHODS

All animals were housed under a 12 hr/12 hr light-dark cycle at the Kansai Medical University Animal Center. Animal experiments were designed and conducted in compliance with the "Guiding Principles for the Care and Use of Laboratory Animals" approved by The Japanese Pharmacological Society.

Culture of RPE cells

Eyes were enucleated from albino rabbits under ether anesthesia, immersed in Ca²⁺- and Mg²⁺-free Hanks' solution supplemented with 10 mM N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) (HEPES) and kept overnight in the dark at 4°C. The eyes were cut at the equator, and the anterior segments and neural retinas were removed. The posterior eyecups were filled with 1.1% trypsin in phosphate-buffered saline (PBS) containing 0.02% ethylenediaminetetraacetic acid (EDTA). After incubation at 37°C for 30 min, the trypsin solution was replaced with horse serum. The RPE cells were dissociated by gentle pipetting with a Pasteur pipette, collected in culture medium [Dulbecco's modified Eagle's medium (DMEM) supplemented with 7% each of fetal calf serum (FCS) and horse serum (HS)] and centrifuged at 80 x g for 8 min. The cells were then suspended in the culture medium, seeded at 1 x 10⁵ cells/35-mm plastic dish and cultured at 37°C in moist 5% CO₂/95% air. The primary cultures were grown to confluence (7–10 days) and passaged at a ratio of 1:3. The cells from passages 2 to 4 were grown on plastic dishes for 3 days or, where needed to achieve polarization, on 24.5-mm microporous membrane filters (Transwell-clear insert chambers, 0.4-um pore size; Corning Costar Co., Cambridge, MA, USA) until confluence and then were used for experiments. The purity of the culture was assessed by immunocytochemistry using antibody against RPE cell-specific cytokeratin, cytokeratin 8.13 (Sigma Chemical Co., St. Louis, MO, USA).

RT-PCR cloning of rabbit NPR-A and NPR-B cDNAs

Based on the sequences of rat NPR-A and NPR-B cDNAs, we carried out the cloning of the respective cDNA fragments in rabbits. Total RNAs were extracted from rabbit kidney (NPR-A) and cultured rabbit RPE cells (NPR-B) with TRIzol Reagent (Gibco BRL, Rockville, MD, USA) according to the manufacturer's instructions. The cDNA fragments of rabbit NPR-A and NPR-B were amplified by RT-PCR using the Access RT-PCR System (Promega Co., Madison, WI, USA). Primer sets were designed based on the rat NPR-A cDNA (21) (sense primer, 5'-AATCCACCAGGAGTTCGAGGG, positions 955–974; anti sense primer, 5'-CGGTGTCCCGATCTCCGTTTC, positions 1506–1526) and rat NPR-B cDNA (22) (sense primer, 5'-CAGCTCATCAGAGCGCAACGGCC, positions 715–736; antisense primer, 5'-CTTCCCGGGCTCTGATCGACG, positions 1009–1030). The amplified fragments were TA-cloned into pCR2.1 (Invitrogen, San Diego, CA, USA) and transformed into E. coli INVαF' cells. Sequencing of the inserts was carried out with an ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit (ABI/Percin Elmer, Foster City, CA, USA) using a model 373A ABI PRISM DNA sequencer.

Primers

The primer sets for amplification of rabbit NPR-A and NPR-B cDNAs were designed based on the respective partial sequences determined as described above: for NPR-A, sense primer, 5'-GGCTGCAAGATCATTACGTATAA (corresponding to the rat NPR-A sequence, 5'-GGCTGCCAAA*ATT*ATTAC*TAC*AAA; *, different nucleotide between rabbit and rat) and antisense primer, 5'-ACCTTGGAGCGTTCCGTTCC (corresponding to the rat same sequence) (size of the product: 252 bp) were used; for NPR-B, sense primer, 5'-AGAGGGAGAACCTGACCAACGG (corresponding to the rat NPR-B sequence, 5'-AGAGGGAGAAGCTGACCAAT*GG) and antisense primer, 5'-ACATTTGGAAATTTGTGATACTC (corresponding to the rat sequence, 5'-GGCG*TTCCTGAAC*TTGTGATTCTCG) (size of the product: 215 bp) were used. For amplification of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), a Control Amplifier Set (size of the product: 452 bp; Clontech Lab., Inc., Palo Alto, CA, USA) was used.

Relative quantitation of mRNA by a fluorescence sample reader system

The rabbit RPE cells grown on plastic dishes or on
microporous membranes were rinsed with PBS, and the total RNA was extracted using TRIzol Reagent. The relative quantitations of the levels of expression of mRNAs were carried out essentially by the kinetics analysis of PCR (23). The RNA samples were subjected to RT-PCR using the Access RT-PCR System. The complete reaction mixture (100 µl) containing 4–8 µg total RNA was divided into 5–10 µl aliquots in 0.2-ml PCR tubes. The PCR reaction was performed under the following conditions: reverse transcription at 48°C for 45 min, followed by 2 min of incubation at 94°C, and immediately by PCR amplification using a step-cycle program of 94°C for 30 sec, 62°C for 30 sec and 68°C for 30 sec. The PCR products were removed at 1- to 2-cycle intervals, and all samples were electrophoresed on 2% agarose gels. The gels were stained with a fluorescent dye, Vistra Green (Amersham Pharmacia Biotech, Ltd., Bucks, England), and the fluorescence intensity of each product was measured by a fluorescence sample reader system, FluorImager (Molecular Dynamics, Sunnyvale, CA, USA). The intensities were plotted against the number of PCR reaction cycles on semi-logarithmic scales. The relative original mRNA levels were estimated from the extrapolated points on the y-axis of the linear portion of the curves.

cDNA probes preparation
cDNA probes were labeled with digoxigenin 11-dUTP using a dNTP mixture containing the labeled dUTP (DIG DNA labeling mixture; Boehringer Mannheim GmbH, Mannheim, Germany). Digoxigenin 11-dUTP was incorporated into the second PCR amplified fragment using the target cDNA that was the first fragment amplified by RT-PCR. The PCR mixture for labeling (50 µl) contained each primer at 1 pM; 1 ng target DNA; 200 nM each of dATP, dGTP, dCTP and dTTP + labeled dUTP; and 2.5 units Taq polymerase in 50 mM Tris-HCl (pH 8.0), 50 mM KCl, 1.5 mM MgCl₂ and 0.01% gelatin. The amplified fragments were purified using a Sephadex G-50 column (Quick Spin Column, Boehringer Mannheim GmbH).

Northern blot analysis
Five to ten micrograms of denatured total RNA was applied per lane and separated by electrophoresis on 1% agarose-formaldehyde gels. After 2 time-wash of the gel with 20× SSC (1× SSC: 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0), the RNA was transferred to positively charged nylon membranes (Boehringer Mannheim GmbH) by capillary blotting with 20× SSC for 15–17 hr. The RNAs were fixed by UV cross-linking at 254 nm (0.12 J/cm²). Membranes were prehybridized at 42°C for 1–2 hr in hybridization solution [50% formamide, 5× SSC, 50 mM sodium phosphate, pH 7.4, 2% blocking reagent (Boehringer Mannheim GmbH), 50 µg/ml salmon testis DNA, 0.5% sodium dodecylsulfate (SDS)]. Heat-denatured digoxigenin-labeled probe and dextran sulfate were added to the hybridization solution at final concentrations of 12 ng/ml and 5%, respectively. The incubation was then continued for 16 hr. The blots were rinsed briefly and washed twice at 65°C for 20 min each in 1× SSC, 5% SDS. The hybridized probes were detected using alkaline phosphatase-conjugated anti-digoxigenin Fab fragments (Boehringer Mannheim GmbH) diluted 1:10000 and 0.26 mM disodium 3-(4-methoxyxsiro(1,2-dioxetane-3,2'-5'-chloro)tricycl[3.3.1.1³⁷]decan)-4-yl) phenyl phosphate (CSPD) (Boehringer Mannheim GmbH), a chemiluminescent substrate, according to manufacturer's instructions. The membrane sealed in a hybridization bag was exposed to Kodak-OMAT AR film. To standardize for the amount of the total RNA, the membranes were reprobed using GAPDH cDNA probes. The relative signal intensity was measured by densitometric scanning of the film. Alternatively, the hybridized bands were detected by a fluorescent alkaline phosphatase substrate (Atoxophos substrate, Amersham Pharmacia Biotech Ltd.), and their fluorescence intensities were measured using FluorImager.

[^H]Thymidine incorporation
RPE cells from passages 2 to 4 were plated in 16-mm wells at 6 × 10⁴ cells/well and grown for 2 days in DMEM supplemented with 7% each of FCS and HS. They were next washed and incubated in serum-free DMEM for 24 hr and then exposed to various factors or transmitters in DMEM supplemented with 2% FCS or to the medium alone for 20 hr. The cells were then pulsed with 2 µCi/ml[^H]thymidine (16.3 Ci/mmol; New England Nuclear, Boston, MA, USA) for 2 hr. After removal of the medium, the cells were treated with ice-cold 5% trichloroacetic acid (TCA) for 10 min and the acid-insoluble fraction was sedimented in a microcentrifuge. The resulting pellets were washed five times with ice-cold 5% TCA and solubilized in 0.3 ml of 1 N NaOH containing 0.1% SDS for 1 hr. Fifty microliters of the suspensions were then added to 5 ml of scintillation cocktail, Clear Sol II (Nacalai Tesque, Kyoto), and the radioactivities were counted in a scintillation counter (Packard Series 4530 Tri-Carb, 43% efficiency; Packard Instrument Co., Inc., Meriden, CT, USA). Aliquots of the suspensions were also assayed for protein content by the method of Lowry et al. (24) and the results were expressed as d.p.m./µg protein. All values (mean±S.E.M.) were analyzed using analysis of variance (ANOVA) and statistical comparisons were made by Student’s unpaired t-test. A P-value less than 0.05 was taken as significant.
Fluorometric measurement of changes in intracellular Cl⁻ concentration

The cultured RPE cells were loaded with a Cl⁻-sensitive fluorescent probe, N-6-methoxyquinolylacetoethyl ester, and the intracellular Cl⁻ concentration was measured as previously described (16).

Drugs

ANP and CNP (human) were obtained from Peptide Institute Inc., Osaka. Melatonin was from Aldrich Chem Co., Milwaukee, WI, USA. Platelet-derived growth factor (PDGF) was purchased from Becton Dickinson Labware, Bedfold, MA, USA. Recombinant human basic fibroblast growth factor (bFGF) was from R&D Systems, Inc., Minneapolis, MN, USA. Transforming growth factor-β1 (TGF-β1) was obtained from Gibco. Other chemicals were of the highest quality commercially available.

RESULTS

Demonstration and relative quantitation of expression of NPR-A and NPR-B mRNAs

To design primer sets for RT-PCR and probes for Northern blotting, we determined the sequences of rabbit NPR-A and B cDNA fragments (NPR-A, 533 bp; NPR-B, 274 bp) by cloning: the sequence of rabbit NPR-A shared 83.6% identity in nucleotide sequence and 81.2% identity and 94.9% similarity in the deduced amino acid sequence, with the corresponding region of rat NPR-A. The sequence of rabbit NPR-B shared 90.2% identity in nucleotide sequence and 99.0% identity in the deduced amino acid sequence with the corresponding region of rat NPR-B.

Northern blot analysis revealed major approximately 4.0-kb bands for NPR-A and NPR-B mRNAs, with an apparently higher level of NPR-B mRNA (Fig. 1). These molecular sizes were similar to those previously observed in other tissues of rat (5, 11, 22) or human (25).

RT-PCR using specific primer sets and total RNA from cultured rabbit RPE cells resulted in the amplification of single bands of the expected sizes (252 bp, NPR-A; 215 bp, NPR-B). We compared the expression levels of NPR-A and NPR-B mRNAs by semi-quantitative RT-PCR. Figure 2A shows representative electrophoretic patterns showing the yield of RT-PCR products at the indicated numbers of reaction cycles, as detected by a fluorescent dye. When the intensity of fluorescence was plotted on semi-logarithmic graphs, the linear portions of the two curves were parallel to each other (Fig. 2B), suggesting that the efficiencies of both PCR reactions were close to each other. The relative levels of NPR-A and NPR-B mRNAs were therefore calculated as described in Materials and Methods. Similar experiments were repeated for RPE cells from separate cultures, and the expression level of the NPR-B mRNA was calculated to be 10.0 ± 0.9-fold (n = 3) higher than that of NPR-A mRNA in RPE cells.

Effects of various treatments of RPE cells on the expression of NPR-A mRNA

We examined the effects of various treatments of RPE cells that may change subretinal fluid absorption on the level of expression of the NPR-A mRNA (Fig. 3). The subretinal fluid absorption, which occurs in the vitreous-to-choroid direction, alters between the light and dark. The light onset stimulates endogenous dopamine release from a variety of vertebrate retinas (26, 27), and dopamine mimics the effect of light on a number of retinal processes (28, 29). On the other hand, melatonin, which is known to be increased in the dark in photoreceptors (30), promotes some dark-adaptive responses in the retina (31, 32). RPE cells were grown to confluence on microporous membranes and treated for 20 min on the apical and basal side with 1 μM each of dopamine, melatonin or norepinephrine, a catecholamine known to be released from retina without significant difference in the light or dark (27). As shown in Fig. 3A, no drug significantly changed the expression of the NPR-A mRNA. Similarly, levels of the expression of GAPDH mRNA as a reference were not changed with these drugs (data not shown). Subretinal fluid absorption is reportedly accelerated by systemic injection of acetazolamide or hyperosmotic solution (33, 34). We therefore treated RPE cells with 1 mM acetazolamide on the basal side for 20 min and with 25 mM mannitol solution on the apical or basal side for 60
Fig. 2. Relative quantitation of NPR-A and NPR-B mRNAs by PT-PCR. A: Representative electrophoretic patterns of the RT-PCR products for NPR-A and NPR-B mRNAs. To analyze each mRNA, 8 µg of total RNA from RPE cells was subjected to RT-PCR (100 µl), and 5.5-µl aliquots of the product were removed at 1- to 2-PCR cycle intervals. All samples were loaded onto 2% agarose gels and visualized using a fluorescent dye. B: Representative determination of relative initial amounts of mRNA according to kinetics analysis of RT-PCR. The fluorescence intensity of each band was measured and plotted on a semi-logarithmic graph. ○, RT-PCR product for NPR-A; ●, RT-PCR product for NPR-B.

Fig. 3. Quantitative RT-PCR for NPR-A mRNA. A: Effects of dopamine, melatonin and norepinephrine on the expression of NPR-A mRNA. RPE cells grown to confluence on microporous membranes were incubated with medium alone (○) or with 1 µM dopamine (□), melatonin (△) or norepinephrine (○) for 20 min, and 8 µg of each total RNA was subjected to quantitative RT-PCR for NPR-A mRNA as described in "Materials and Methods". B: Effect of acetazolamide and hyperosmotic mannitol solution on the expression of NPR-A mRNA. RPE cells grown to confluence on microporous membranes were treated with medium alone (●) for 20 min, 1 mM acetazolamide on the basal side (■) for 20 min, or with 25 mM mannitol on the apical (▲) or basal side (◆) for 60 min, and 8 µg of each total RNA was subjected to quantitative RT-PCR for NPR-A mRNA.
min. No treatment changed the expression of NPR-A mRNA (Fig. 3B) as well as that of GAPDH mRNA (data not shown). Similar results were obtained in three repetitions of these experiments and by Northern blot analysis (data not shown). These findings suggest that NPR-A mRNA expression in RPE cells may not be regulated by a light-dark transition nor by ionically or osmotically mediated changes in subretinal fluid absorption.

**Effects of various treatments of RPE cells on the expression of NPR-B mRNA**

The function of CNP or its receptor, NPR-B, in RPE cells has not been characterized. Our study using a Cl\textsuperscript-- sensitive fluorescent dye showed that CNP (10\textsuperscript{-11} - 10\textsuperscript{-7} M) did not affect the intracellular Cl\textsuperscript-- concentration in RPE cells (10\textsuperscript{-11} M, 98.5±0.5% of control; 10\textsuperscript{-10} M, 99.1±0.6%; 10\textsuperscript{-9} M, 100.1±0.8%; 10\textsuperscript{-8} M, 98.3±1.2%; 10\textsuperscript{-7} M, 98.3±0.4%; n = 4). We next examined whether the expression of NPR-B mRNA is regulated by the treatments affecting the light- or dark-adaptation or the subretinal fluid transport (Fig. 4). Figure 4A shows the effects of dopamine, melatonin and norepinephrine on the levels of expression of NPR-B mRNA in RPE cells, as analyzed by Northern blotting. The addition of dopamine or melatonin significantly decreased the expression (dopamine, 61.1±13.8% of control; melatonin, 77.7±9.6% of control). Figure 4B shows the effect of application of acetazolamide on the basal side and hyperosmotic mannitol solution on the apical or basal side on the expression of NPR-B mRNA. Only the application of acetazolamide showed a slight tendency to increase the expression (107.6±7.5% of control; P < 0.10, n = 5).

**Effects of ANP, CNP and growth factors on proliferation of RPE cells**

To clarify whether ANP and CNP regulate the proliferation of RPE cells, RPE cells in subconfluent states were treated with ANP or CNP in the presence of 2% FCS for 20 hr, and DNA synthesis was estimated by measuring [\textsuperscript{3}H]thymidine incorporation (Fig. 5). CNP

![Graph](image)

**Fig. 4.** Northern blot analysis for NPR-B mRNA. A: Effect of dopamine, melatonin and norepinephrine on the expression of NPR-B mRNA. B: Effect of acetazolamide and hyperosmotic mannitol solution on the expression of NPR-B mRNA. RPE cells grown to confluence on microporous membranes were treated as described in Fig. 3A or 3B, and 10 μg of each total RNA was subjected to Northern blotting using the digoxigenin-labeled cDNA probe for NPR-B. Bars represent the mean±S.E.M. (n = 3–5). **P < 0.01, *P < 0.05, by Student's unpaired t-test for the difference from the control.
Fig. 5. Effects of ANP and CNP on the proliferation of RPE cells. RPE cells were grown for 2 days after plating, incubated in serum-free medium for 24 hr and then exposed to medium alone (control) or to various concentrations of ANP (right) or CNP (left) in medium containing 2% FCS for 20 hr. The cells were pulsed with 2 μCi/ml[^3]H]thymidine for 2 hr and the incorporation of the radioactivity was determined as described in "Materials and Methods". Bars represent the mean±S.E.M. (n=4–9). **P<0.01, by Student’s unpaired t-test for the difference from the control.

Fig. 6. Effects of PDGF, TGF-β, bFGF, dopamine, melatonin, norepinephrine and acetazolamide on the proliferation of RPE cells. RPE cells were grown for 2 days after plating, incubated in serum-free medium for 24 hr and then exposed to medium alone (control); 10 ng/ml PDGF; 10 ng/ml TGF-β; 10^-7 M bFGF; 1 μM dopamine, melatonin or norepinephrine; or 1 mM acetazolamide in medium containing 2% FCS for 20 hr. The cells were pulsed with 2 μCi/ml[^3]H]thymidine for 2 hr and the incorporation of the radioactivity was determined. Bars represent the mean±S.E.M. (n=5–6). **P<0.01, by Student’s unpaired t-test for the difference from the control.

significantly and dose-dependently suppressed the incorporation, with maximal suppression to 39.4±3.9% of the control level at 10^-4 M. On the other hand, ANP showed a significant but biphasic effect; in the lower dose range (10^-12–10^-10 M), it strongly inhibited, with maximal suppression to 32.3±7.8% of the control level at 10^-10 M, but in the higher dose range, its inhibitory effect was attenuated.

Growth factors such as PDGF, TGF-β and bFGF have been shown to play a role in some retinal pathogenesis or wound healing by affecting growth or proliferation of RPE cells (35, 36). We further examined the effects of these growth factors as well as the drugs used in the experiments described above on the proliferation of RPE cells in the subconfluent state. As shown in Fig. 6, DNA synthesis was significantly promoted by 10 ng/ml PDGF (254.0±76.9% of control) and significantly inhibited by melatonin (39.0±8.3% of control).

Effects of ANP, CNP, PDGF, TGF-β and bFGF on the expression of NRP-B mRNA

To examine whether the expression of NRP-B mRNA is regulated by growth factors, RPE cells in the subconfluent state were exposed to 10^-10 M ANP, 10^-8 M CNP, 10 ng/ml PDGF, 10 ng/ml TGF-β or 10^-9 M bFGF for 22 hr, and the levels of expression of NRP-B mRNA were compared by Northern blot analysis (Fig. 7). The expression was significantly decreased by CNP, PDGF and TGF-β, most markedly by PDGF (58.8±5.9% of control) and to a lesser extent by TGF-β (64.0±9.0% of control) and CNP (85.8±4.7% of control). Such signifi-
significant inhibition was not seen in Northern blot analysis for NPR-A mRNA, which was carried out in parallel (data not shown).

DISCUSSION

In the present study, we demonstrated the presence of NPR-A and NPR-B mRNAs in cultured RPE cells, with a markedly higher level of the NPR-B mRNA. Since the expression of NPR-A mRNA was not altered by treatments affecting the transport of subretinal fluid, it appeared that NPR-A mRNA might be constitutively expressed in the RPE, irrespective of the regulatory change of subretinal fluid absorption. On the other hand, factors affecting cell proliferation or growth suppressed the expression of NPR-B mRNAs, and both ANP and CNP inhibited the proliferation of RPE cells, suggesting that the NP system in the RPE plays a role in the pathophysiological regulation of cell proliferation. This is the first study describing the expression of the NPR-A and NPR-B mRNAs in RPE cells and the effects of the NP system on the proliferation of the RPE cells.

We previously showed that ANP stimulates the Na⁺/K⁺/2Cl⁻ cotransporter in RPE cells through the activation of guanylate cyclase (16). The Na⁺/K⁺/2Cl⁻ cotransporter mediates active absorption of Cl⁻ across the apical membrane which then flows down its electrochemical gradient across the basal membrane Cl⁻ channels. This mechanism contributes to subretinal fluid absorption by driving fluid across the apical membrane (17), and the absorption may be one of the mechanisms responsible for maintaining proper retinal adhesion. The subretinal fluid absorption is thought to be changed physiologically in the light- and dark-adaptation and increases experimentally by intravenous injection of acetazolamide (34) or hyperosmotic mannitol solution (33). The present study suggested that the NPR-A mRNA may be constitutively expressed irrespective of the regulatory changes of the subretinal fluid absorption. It is therefore possible that the expression of ANP mRNA in RPE cells or the choroid might be altered in the regulation of subretinal fluid transport, although the expression of ANP mRNA has not been analyzed in the RPE. Alternatively, the ANP system in RPE cells may contribute to constitutive subretinal fluid absorption but not to regulated absorption.

It has been shown that cAMP inhibits subretinal fluid absorption and cGMP stimulates it (34). Therefore, in the light, dopamine might stimulate subretinal fluid absorption through the activation of D₁ dopaminergic receptors on RPE (29) and the subsequent decrease in intracellular cAMP concentration. In bovine RPE, it has been shown that apical epinephrine stimulates subretinal fluid absorption via a mechanism including an apical membrane α₁ adrenergic receptor, apical membrane Na⁺/K⁺/2Cl⁻ cotransporter and basolateral membrane Cl⁻ conductance (17). Such a mechanism may also be involved in the light-dependent change of subretinal fluid absorption.

CNP suppressed the proliferation of the cells (Fig. 5) without effects on the Cl⁻ transport activity in RPE cells. Thus, the CNP system appeared to function mainly as a regulator of cell proliferation in RPE cells. Since CNP mRNA has been detected in retina (12), the change in the level of NPR-B mRNA in RPE cells might affect the paracrine effect of CNP on the cells. The expression of NPR-B mRNA was suppressed by the addition of dopamine or melatonin (Fig. 4). This, together with the observation that melatonin significantly inhibited the cell proliferation (Fig. 6), might mean that the proliferation of RPE cells is also oppositely regulated by the light-dark adaptation. In the light, dopamine may attenuate the inhibitory effect of CNP on the cell proliferation via suppression of the NPR-B mRNA level. On the other hand, in the dark, melatonin itself can suppress the prolifera-
tion while simultaneously attenuating the inhibitory effect of the other system, the CNP system.

The present results support the recent notion that ANP as well as CNP have growth-regulatory properties. Depending on the cell types, ANP and/or CNP show growth-regulatory properties in various tissues such as osteoblastic cells, glomerular mesangial cells, vascular smooth muscle cells, aortic endothelial cells and chondrocytes (18, 19, 37). In RPE cells, both ANP and CNP inhibited the proliferation, but the manners of inhibition were different (Fig. 5). CNP dose-dependently suppressed the proliferation, while ANP had strong and dose-dependent inhibitory effects in the lower dose range of $10^{-12}$–$10^{-10}$ M, with maximal effect at $10^{-10}$ M, but the effect was attenuated in the higher dose range of $10^{-9}$–$10^{-7}$ M. At the lower concentrations, ANP promoted the accumulation of [Cl$^-$] in RPE cells, and no attenuation of the effect on [Cl$^-$] was observed at the higher doses (16). The cellular mechanism involved in the growth-regulatory actions of the NPs are not totally clear. There are evidence supporting the involvement of both NPR-A and NPR-C for ANP effects (18), cGMP-dependent and cGMP-independent mechanisms (38), and cytosolic calcium (39). In addition, higher doses of a given NP stimulate the receptor of the other NPs (5). Thus, interactions between these cellular mechanisms or between NPRs might cause the attenuation of ANP effect at higher doses. Further studies may be necessary to clarify the cellular mechanism responsible for the proliferation-regulatory action.

A number of pathological states, including trauma, inflammation, retinal detachment, and proliferative vitreoretinopathy, initiate a sequence of cellular responses controlled by growth factors (40). Proteins such as PDGF, TGF-$\beta$ and FGF affect cellular migration and proliferation or growth. There are some evidence showing the counter-regulatory role of the NP system in the processes mediated by growth factors. In vascular smooth muscle cells, ANP inhibits PDGF-stimulated proliferation (41) via inhibition of PDGF receptor tyrosine kinase activation (42) and attenuates the increase in the synthesis of RNA and protein caused by TGF-$\beta$, a potent hypertrophic factor (43). In our study, PDGF potently promoted the proliferation of RPE cells in the subconfluent state, while TGF-$\beta$ did not. Interestingly, PDGF and TGF-$\beta$ inhibited the expression of NPR-B mRNAs. Since the expressions of NPR-B and CNP are concomitantly regulated (44, 45) in various tissues, the change in the level of NPR-B mRNA might contribute at least in part to the modulation of the CNP effects. Thus, RPE cells appear to have counter-acting regulatory mechanisms between growth factors and the NP system.

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