Inhibition of NHE-1 Na\(^+\)/H\(^+\) exchanger by natriuretic peptides in ocular nonpigmented ciliary epithelium

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Natriuretic peptides (NPs) are a family of bioactive polypeptides that includes three different prohormones: atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), and C-type natriuretic peptide (CNP). These peptides display hypotensive effects in the mammalian eye by lowering the intraocular pressure (IOP), a function that is mediated by the bilayer ocular ciliary epithelium (CE), in conjunction with the trabecular meshwork. ANP regulates Na\(^+\)/H\(^+\) exchanger (NHE) activity, and inhibitors of NHE have been shown to lower IOP. We examined whether NPs influence the NHE activity of the CE, which is comprised of pigmented (PE) and nonpigmented (NPE) epithelial cells, by directly recording the rate of intracellular pH (pHi) recovery from its inner NPE cell layer. NPs inhibited, in a dose-dependent manner (1–100 nM), the rate of pHi recovery with the order of potency CNP > ANP > BNP, indicative that this inhibition is mediated by the presence of NPR type B receptors. 8-Bromo-cGMP (8-BrcGMP), a nonhydrolyzable analog of cGMP, mimicked NPs in inhibiting the rate of Na\(^+\)−dependent pHi recovery. In contrast, ethylisopropyl amiloride (EIPA, 100 nM) or amiloride (10 μM) completely abolished the pH\(_i\) recovery by NHE.

Aqueous humor formation is a complex process of ion, water, and protein transport through the CE in a transcellular and paracellular manner. Net secretion of the aqueous fluid is considered to occur toward the posterior chamber in the pars plana region of the CE. However, it was also recently suggested that this secretion might take place in the pars plicata region of the CE. Human aqueous humor BNP and ANP have been found in both healthy and glaucomatous eyes, with elevated levels in the former (35, 36). In the rabbit glaucoma eye model (buphthalmus), down-regulation of NPR receptors has been reported in ciliary processes (16).

Aqueous humor is a complex process of ion, water, and protein transport across the CE. The current understanding of crucial ionic transport mechanisms involved in aqueous humor secretion includes a variety of channels and transporters, including the paired Na\(^+\)/H\(^+\) exchanger (NHE) and Cl\(^-\)/HCO\(_3\)\(^-\) exchangers (AE), the bu- metanide-sensitive Na\(^+\)/K\(^+\)−2Cl\(^-\) cotransporter, and Na\(^+\)/K\(^+\)− ATPase (5).

Each of these ion transport proteins is believed to play a role in the modulation and maintenance of IOP. Recently, Avila et al. (3) found that inhibitors of the NHE lower IOP when applied to anesthetized mice. Little, however, is known about activity in a cGMP-independent fashion. The NP binding of NPR-A and NPR-B causes the generation of intracellular cGMP that leads to the activation of a protein kinase G (PKG). Downstream kinase activities include regulation of ion channels, substrate protein phosphorylation, and cellular proliferation (26).

In glaucoma, elevation of the intraocular pressure (IOP) has been reported to be an important risk factor in the development of the disease, one of the leading causes of blindness in the world (41). A balance between aqueous humor secretion by the ciliary epithelium (CE) and its drainage throughout the trabecular meshwork regulates IOP. Previous findings indicate that NPs reduce IOP in several species including monkey, rabbit, and bovine (14, 37, 39). In clinical studies, a hypotensive effect has been reported in humans and in experimental animals with elevated IOP (14, 20, 48). It is generally believed that NPs exert their effects on IOP by reducing the rate of aqueous humor secretion by the CE, although involvement of the trabecular meshwork and the outflow pathway has also been proposed (7, 32). To date, many of the components that comprise the NP system have been identified in the CE, including NPs and their cognate receptors (15, 31). In the aqueous humor ANP and BNP have been found in both healthy and glaucomatous eyes, with elevated levels in the former (35, 36). In the rabbit glaucoma eye model (buphthalmus), down-regulation of NPR receptors has been reported in ciliary processes (16).

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the nature of the endogenous effector molecules that regulate IOP, and in particular whether NPs may serve as signaling molecules in this mechanism. Earlier studies have been suggestive of the effect of NPs on Na\(^+\) and K\(^+\) channels and their inhibitory effect on aquaporin channels (4, 23). In kidney and heart, NPs influence ion transport activities of the NHE and/or Na\(^+-\)K\(^+\)-2Cl\(^-\) cotransporter (26). These transport systems are also believed to contribute to fluid secretion in the CE. In the present study we examined the physiological role of NPs on the NHE expressed in the ocular CE. The NHE are among the major transporters involved in cell volume regulation. Their activation leads to a cellular influx of Na\(^+\) and extrusion of H\(^+\), resulting in a net import of Na\(^+\). In many systems NHE functions in parallel to AE, resulting in the uptake of NaCl and osmotically obliged water. The export of protons may link NHE activation to changes in intracellular pH (pHi), and the NHE can independently respond to alterations of pHi and cell volume. NP has been shown to either stimulate or inhibit NHE.

**MATERIALS AND METHODS**

All studies were approved by the Yale University Animal Care and Use Committee and followed the “Guiding Principles for Research Involving Animals and Human Beings” of the American Physiological Society.

**Source of tissue.** Bovine eyes were obtained from 6- to 12-wk-old calves through a local abattoir, and rat eyes were enucleated from Sprague-Dawley rats weighing 150–250 g (Charles River Laboratories, Wilmington, MA). Single strips of ciliary processes from the pars plicata region of the CE were microdissected from the ciliary body as previously described (18).

**Chemicals and solutions.** The following solutions were used in perfusion experiments (in mM): 1) HEPES-buffered Ringer solution: 115 NaCl, 5 KCl, 1 CaCl\(_2\), 1.2 MgSO\(_4\), 2 NaH\(_2\)PO\(_4\), 32.2 HEPES, and 10 glucose; 2) HEPES-buffered NH\(_4\)Cl solution: 20 NH\(_4\), 95 NaCl, 5 KCl, 1 CaCl\(_2\), 1.2 MgSO\(_4\), 2 NaH\(_2\)PO\(_4\), 32.2 HEPES, 10 glucose, and 8 mannitol; 3) HEPES-buffered Na\(^+\)-free solution: 122 NaH\(_2\)PO\(_4\), 2 KH\(_2\)PO\(_4\), 32.2 HEPES, 10 glucose, and 15 mannitol. All solutions were preheated to 37°C and adjusted to a pH of 7.4 before the experiment. The following drugs (obtained from Sigma, St. Louis, MO) were added to the solutions at concentrations indicated in Figs. 2-5: ANP, CNP, amiloride, bumetanide, benzamil, 8-bromo-cGMP (8-BrcGMP), lysophosphatidic acid (LPA) 18α-glycerethenic acid (18α-GA). BNP was purchased from Bachem (Bubendorf, Switzerland), ethylisopropyl amiloride (EIPA) and 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein were obtained from Molecular Probes (Eugene, OR), HOE-694 was a gift from Carsten Wagner (University of Zurich, Zurich, Switzerland), and A-79195 was a gift from Abbott Laboratories. With the exception of bumetanide, which was added after the NH\(_4\) pulse (because of inhibition of NH\(_4\) uptake; see Fig. 1), all drugs were added over the entire course of the experiment. The concentration of solvent reagents used for dissolving the drugs (dimethyl sulfoxide or ethanol) never exceeded 0.1%.

**Reverse transcription-polymerase chain reaction.** Total RNA was isolated from bovine processes and from cultured ciliary pigmented epithelial (PE) and NPE cells with TRIzol reagent (Invitrogen, Carlsbad, CA), and cDNA was synthesized in vitro with a reverse transcription-polymerase chain reaction (RT-PCR) kit (Stratagene, La Jolla, CA). PCR was carried out with sets of primers based on published cDNA nucleotide sequences of bovine NHE-1 (GenBank accession no. AJ131763) and NHE-3 (GenBank accession no. AJ131764). The oligonucleotide primers used in PCR were forward: (nt 47–69) 5'-TCAAATGGGAGCA GCCGTA GT-3' and reverse: (nt 389–405) 5'-CAAGGGCGCATTGCTA-3' for NHE-1 and forward: (nt 65–88) 5'-GTGATCAAGGCCCATTGAGGT-5' and reverse: (nt 605–624) 5'-TTGTCGTGCTGCGGG-GAACG-3' for NHE-3. The expected DNA size products in PCR reactions were 359 bp for NHE-1 and 560 bp for NHE-3. The predicted cDNA products amplified by PCR were gel purified and sequenced in an automated DNA sequencer ABI PRISM 310 Genetic Analyzer, with the rhodamine terminator cycle sequencing ready method (PE Applied Biosystems, Foster City, CA). Nucleotide sequences were aligned and verified to share 100% homology with the corresponding published bovine cDNA sequences in GenBank.

**pHi measurements and BCECF fluorescence imaging.** Freshly microdissected ciliary processes from bovine and rat eyes were placed on top of coverslips precoated with CellTak tissue adhesive (Bioscience, Bedford, MA) and incubated with 10 M BCECF-acetoxyethyl ester (Molecular Probes) for 10 min. Coverslips were then transferred to a preheated (37°C) perfusion chamber on an Olympus IX70 inverted microscope with a ×20 objective and washed with HEPES-buffered Ringer solution to remove any residual deesterified dye. After excitation at 490 and 440 nm with a Lambda DG-4 light source (Sutter Instrument, Novato, CA), the fluorescence signal emission was recorded at 535 nm with a Quantix camera (Roper Scientific, Tucson, AZ). Recordings were made from the NPE cell layer only because the PE layer did not show an uptake of the BCECF dye and the pigment absorbed the fluorescent signal. Data was collected every 15 s and processed with MetaFluor software (Universal Imaging, Downingtown, PA). pHi was calculated from the 490 nm-to-440 nm intensity ratio with the high K\(^+\)/nigericin calibration method (40).

In the course of the experiment the cells were acidified by perfusion with NH\(_4\)Cl-HEPES-buffered solution for 4 min followed by Na\(^+\)-free HEPES solution. After readdition of HEPES-buffered Ringer solution the realkalization rate was monitored to calculate Na\(^+\)-dependent pHi recovery. NHE activity was calculated as change of pHi per minute (ΔpHi/min) from the gradient during Na\(^+\)-dependent pHi recovery. Because the activity of NHE transporters is pH dependent (2) and the cells varied in pHi after acidification, we chose a fixed pHi value for all cells that was used as a start point for calculation of activity and thus avoided changing intracellular buffering power. The fixed pHi values were 6.80 for bovine NPE cells and 7.04 for rat NPE cells.

**Indirect immunofluorescence.** Cryostat sections (1–2 μm thick) of the bovine and rat CE were prepared as described previously (18). Sections were incubated with polyclonal antibodies against ANP, BNP, or CNP (Phoenix Pharmaceuticals) diluted 1:100 in 10% normal horse serum containing 1% BSA in PBS for 2 h at 37°C in a humidified atmosphere. After being washed with 1% BSA in PBS, the sections were incubated for 1 h at 37°C with the secondary antibody (rhodamine-conjugated anti-rabbit immunoglobulin) followed by three 10-min washes in PBS. The sections were mounted in a solution of glycerol (pH 7.0) and analyzed in a Zeiss microscope equipped with epifluorescent microscopy. Antibodies against the NHE-1 isofrom were the generous gift of Dr. Sergio Grinstein (Division of Cell Biology, Hospital for Sick Children, University of Toronto, Toronto, ON, Canada) and were characterized previously (21). Antibodies to Na\(^+\)-K\(^+\)-ATPase at a dilution of 1:100 were used as previously described (19).

**Statistical analysis.** Unpaired Student’s t-test was used to determine significance between two experiments; P values <0.05 were considered to be statistically significant. If not otherwise stated, data are given as means ± SE. The number of cells recorded (n) during one experiment varied between 8 and 12, and each experiment was conducted at least four times.
RESULTS

pH$_i$ recording of NPE cells in ciliary epithelium. The pH$_i$ of bovine and rat NPE cells was recorded as described in MATERIALS AND METHODS. Under control conditions, strips of ciliary processes were incubated with pH-sensitive dye (BCECF) and the pH$_i$ was monitored and recorded from single cells in the intact CE under HCO$_3$-free conditions with a digital imaging system. Figure 1 shows a representative profile of the pH$_i$ recorded from a bovine NPE cell in response to an acid load with 20 mM NH$_4$Cl, followed by Na$^+$-free solution and subsequent return to a Na$^+$-containing solution. The rise in pH$_i$ induced by a NH$_4^+$ pulse (phase a) led to an acidification (phase b) in the absence of Na$^+$ (Fig. 1). The addition of Na$^+$ (phase c) resulted in a realkalinization (phase d) and recovery of pH$_i$ to control normal values (Fig. 1).

Under these conditions the mean pH$_i$ in the bovine NPE cells was 7.096 ± 0.018 (n = 64), whereas the mean pH$_i$ was 7.392 ± 0.031 in rat NPE cells (n = 48). After acidification, bovine NPE cells reached a pH$_i$ between 6.90 and 7.10, whereas rat NPE cells showed a pH$_i$ between 6.65 and 6.85. The alkalization rate was calculated on the basis of the fixed set points after readdition of Na$^+$ as indicated in MATERIALS AND METHODS. The alkalization rate was estimated at 0.162 ± 0.005 pH/min (n = 48) in bovine NPE cells and 0.234 ± 0.007 pH/min (n = 48) in rat NPE cells.

Molecular identity and functional characteristics of NHE expressed in bovine NPE cells. To characterize the molecular and functional properties of the NHE subtypes expressed in bovine CE, we carried out RT-PCR on cDNA synthesized in vitro. At least nine NHE isomers have been cloned so far, of which only NHE-1 and NHE-3 bovine sequences are available. Sets of oligonucleotide pair primers were selected for bovine NHE-1 and NHE-3 (see MATERIALS AND METHODS) and annealed to bovine cDNA prepared from bovine ciliary processes and bovine NPE and PE cells. Of the two sets of pair primers used, only the NHE-1 primers amplified a DNA product of the expected size (359 bp) in intact CE and NPE and PE cells (Fig. 2A). No PCR DNA product was amplified with the NHE-3 primers. Nucleotide sequencing of the 359-bp DNA product confirmed that it shared 100% homology with the published cDNA sequence for the bovine NHE-1 isomer.

We next investigated functional properties of the NHE-1 isomer expressed in bovine NPE cells. We found that the NHE-1-specific inhibitor HOE-694 (1 μM) (9, 30) completely inhibited the Na$^+$-dependent pH$_i$ recovery of NPE cells (Fig. 2B). Less specific NHE-1 inhibitors including EIPA (100 nM to 10 μM) and amiloride (10 μM) (24, 25) also inhibited the pH$_i$ recovery in NPE cells at higher concentrations (Fig. 2C).

NPs inhibit Na$^+$-dependent pH$_i$ recovery in bovine and rat NPE cells. Earlier studies indicated that among the physiological effect of NPs figured their ability to inhibit cellular NHE activity (22). Before examining the effect of NPs on NHE-1 activity in bovine NPE cells, we verified whether NP treatment modified the intrinsic buffering capacity of the cells. In the
absence of NP, the start pH, in bovine NPE cells was 7.096 ± 0.018, and in the presence of CNP, the pH, was 7.12 ± 0.020. Similarly, in the presence of ANP, the pH, in bovine NPE cells was 7.14 ± 0.018. When rat NPE cells were analyzed, the start pH, was 7.392 ± 0.031 and pH, in the presence of CNP or ANP was 7.45 ± 0.025 or 7.48 ± 0.010, respectively. These results suggested that NP did not change the buffering capacity of NPE cells significantly. ANP, BNP, and CNP added separately at a concentration of 10⁻⁷ M significantly (P < 0.001) reduced the alkalinization rate in the order of potency CNP > ANP > BNP (Fig. 3A).

CNP (10⁻⁷ M) inhibited the rate of Na⁺-dependent pH, recovery (0.051 ± 0.003 pH/min; n = 40) in a more potent fashion than ANP (10⁻⁷ M: 0.076 ± 0.004 pH/min; n = 28) or BNP (10⁻⁷ M: 0.096 ± 0.006 pH/min; n = 39) (Fig. 3B). Interestingly, this order of potency to inhibit Na⁺-dependent pH, recovery correlates with the NP ability to activate cGMP synthesis in NPE cells (10, 31). This indicates that the NP type B receptor (NPR-B) is the primary receptor mediating NP cellular effects in NPE cells (10, 13). The inhibitory effect of CNP on the rate of Na⁺-dependent pH, recovery in NPE cells was dose dependent within the range of 10 pM to 100 nM (Fig. 4A) with a log EC₅₀ = −8.931 (Fig. 4B).

8-BrcGMP (1 mM), a hydrolysis-resistant cGMP analog, mimicked the effect of NP in bovine NPE cells by inhibiting the rate of Na⁺-dependent pH, recovery at 0.113 ± 0.011 pH/min (n = 12). This level of inhibition was comparable to the inhibitory effect elicited by CNP at 1 nM.

We next tested whether NPs exhibit species differences in their inhibitory effects on NHE activity. For this purpose we determined pH, responses of rat NPE cells to CNP and ANP and compared their effects with those of bovine NPE cells. CNP (10⁻⁷ M) reduced the pH, recovery of rat NPE cells to 0.147 ± 0.005 pH/min (n = 40), whereas ANP (10⁻⁷ M) inhibited the response to 0.187 ± 0.006 pH/min (n = 75). These values were significantly lower (P < 0.001) than the control 0.234 ± 0.007 pH/min (n = 48). However, the magnitude of inhibition of NHE activity by CNP and ANP was significantly higher in bovine NPE cells than in rat NPE cells. Despite the species differences in the inhibitory effect on NHE activity of CNP and ANP, rat NPE cells showed the same phase shift of NHE activity as bovine cells.

To verify whether the rate of pH, recovery in rat NPE cells in the presence of CNP reflects a contribution of NHE activity in PE cells, we used the gap junction blockers 18o-GA and heptanol (17, 30). In the presence of 18o-GA (100 μM) alone, the rate of pH, recovery in rat NPE cells was 0.144 ± 0.013 pH/min (n = 30), whereas in the presence of heptanol (2 mM), it was 0.153 ± 0.005 pH/min (n = 19). However, when 18o-GA was added together with CNP (100 nM), the pH,
recovery was 0.184 ± 0.006 pH/min (n = 47). This indicated that 18α-GA attenuates the inhibitory effect of CNP on the rate of pH recovery. Table 1 summarizes the effect of NP on the pH recovery in bovine and rat NPE cells.

Antagonists of NPR-B receptors can block inhibitory effect of NP on NHE-1. To verify that the inhibitory effect of CNP on NHE-1 activity in NPE cells is mediated by NPR-B receptors, we attempted to block the NHE inhibition by A-71915 and LPA. A-71915, an analog of ANP, is known to exhibit an inhibitory effect on the cGMP-mediated stimulation of ANP and BNP on NPE cells (10, 11, 31). In contrast, LPA has been shown to block the effect of CNP by binding NPR-B (1). In the presence of A-71915 (1 μM), the inhibitory effect of CNP (100 nM) on NHE-1 activity was attenuated by 66.8 ± 3.8% (n = 46) and completely abolished in the presence of LPA (10 μM; n = 42). The addition of both A-71915 and LPA completely reversed the inhibitory effect of CNP on NHE activity (n = 54; Fig. 5). Surprisingly, LPA (10 μM) alone led to a relative activation of NHE-1 up to 29.2 ± 2.7% (n = 36; represented as negative value in Fig. 5), whereas A-71915 (n = 42) alone had no influence on the pH recovery (Fig. 5).

Inhibitors of Na⁺ transport do not influence NHE-1 activity in NPE cells. We studied the influence of other Na⁺ transporters on NHE-1 activity expressed in NPE cells. For this purpose we determined the NHE-1 activity in NPE cells on inhibition of the bumetanide-sensitive Na⁺-K⁺-2Cl⁻ cotransporter or the epithelial Na⁺ channel (ENaC), which have been reported to be present and functional in NPE cells (6, 12). Application of bumetanide (100 μM) or benzamil (10 μM) to bovine NPE cells did not reveal significant differences in pH recovery compared with control (data not shown). Furthermore, the simultaneous addition of bumetanide or benzamil with ANP (100 nM) did not alter the inhibitory effect elicited by ANP alone.

Indirect immunofluorescence of NP and NHE-1 in bovine and rat ciliary processes. We examined the pattern of NP labeling with antibodies to ANP, BNP, and CNP on bovine semithin cryostat sections of ciliary processes. ANP and BNP antibodies preferentially labeled the cytoplasm of the NPE cell layer (Fig. 6, A–D). In contrast, the CNP antibody labeled the vascular endothelium in the stroma of the ciliary processes (Fig. 6, E and F).

We also verified and compared the pattern of immunolabeling of NHE-1 in the CE of bovine and rat ciliary processes. Tissues were incubated with a polyclonal antibody against NHE-1 (21) at a 1:100 dilution and processed as indicated for the NP antibodies. The immunostaining pattern observed indicates that the antibody preferentially labels the basal membrane of NPE cells in the rat (Fig. 7, A and B) and bovine (Fig. 7, C and D) CE. The immunostaining signal at the basal plasma membrane of the PE cells ranged from absent in rat to low in bovine. This pattern of labeling contrasted with the profile of staining with a Na⁺-K⁺-ATPase antibody, which equally labeled both basal membranes in PE and NPE cells in the bovine CE (Fig. 7, E and F). No signal was detected when normal serum or secondary antibody alone was used (Fig. 7, G and H).

**DISCUSSION**

Aqueous humor secretion is a complex process that takes place in the ocular CE, a bilayer of neuroepithelial polarized cells. The present model of aqueous humor secretion contemplates at least three steps: 1) uptake of solute and water at the stromal surface by PE cells, involving a Na⁺-K⁺-2Cl⁻ symport and the parallel Cl⁻/HCO₃⁻ and Na⁺/H⁺ antiports, 2) transfer from PE to NPE cells through gap junctions, and 3) transfer of solute and water from NPE cells into aqueous humor in the posterior chamber of the eye (5). Of the multiple transporters described in the CE the Na⁺/H⁺ antiport (NHE) plays an important role.

NHE-1 is considered a molecular sensor to changes in pH and in cell volume regulation. The NHE catalyzes the electroneutral exchange of extracellular Na⁺ and intracellular H⁺. This results in a net import of extracellular Na⁺ into the cell. In many systems, including the CE, the NHE operates in parallel with a Cl⁻/HCO₃⁻ exchanger, resulting in the uptake of

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<th>Table 1. Effect of NP on pHᵢ and on rate of Na⁺-dependent pHᵢ recovery in bovine and rat NPE cells</th>
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<td>18α-GA (100 μM) + CNP (100 nM) n.d.</td>
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Values are means ± SE for no. of cells in parentheses. NP, natriuretic peptide; pHᵢ, intracellular pH; NPE, nonpigmented epithelium; ANP, atrial NP; BNP, brain NP; CNP, C-type NP; 18α-GA, 18α-glycyrrhetinic acid; n.d., not determined.

Fig. 5. Summary of effects by CNP and NP receptor (NPR) antagonists on NHE-1 activity in NPE cells. The inhibitory effect elicited by CNP (100 nM) on the NHE-1 activity is given a value of 1 and that in the absence of CNP (control of inhibition) a value of 0. In the presence of A-71915 (1 μM), a NPR-A blocker, there is a significant reversal effect on the inhibition of NHE-1 by CNP. However, lysophosphatidic acid (LPA; 10 μM), a NPR-B blocker, completely reversed the effect of CNP when added together (CNP + LPA) or when added with CNP plus A-71915 (CNP + A-71915 + LPA). Interestingly, LPA, but not A-71915, when added alone exhibited a stimulatory effect on NHE-1 activity.
NaCl, and the influx of osmotically obliged water will follow. This effect will consequently lead to an increase in cell volume. Out of the multiple NHE isoforms so far identified, we focused in this study on the NHE-1 that is ubiquitously expressed and highly restricted to the basal membrane of the NPE CE. The diminished (to absent) NHE-1 immunostaining signal along the basal plasma membrane of PE cells on cryostat sections was unexpected, because by RT-PCR amplification NHE-1 mRNA was detected in both cell types of the CE. One possible interpretation of this distinct labeling of NHE-1 in NPE and PE cells is that the epitope that recognizes the NHE antibody is masked in the PE cells. Alternatively, this difference could simply reflect a higher abundance of NHE-1 expression in NPE than in PE cells. Additional antibodies to NHE-1 protein could help to corroborate this finding.

In the present study we attempted to determine whether NPs, which exhibit hypotensive effects in lowering IOP, mediate their effect by regulating NHE activity in the CE. The NPE and PE cells in the CE are coupled to each other by numerous gap junctions (8).

The main effect of NPs on the NHE-1 activity in NPE cells was their ability to inhibit the Na\(^+\)-dependent pH\(_i\) recovery on an acid load. The level of inhibition of NHE-1 by NPs followed an order of potency (CNP > ANP > BNP) that was similar to their ability to stimulate cGMP in cultured NPE cells (31). These results indicate that NPR-B receptors mediate both the inhibition of Na\(^+\)-dependent pH\(_i\) recovery and the stimulation of cGMP in NPE cells. On the basis of the reversing effect of A-71915 on the inhibition of NHE-1 activity by CNP, we could not rule out that NP type A receptor is not involved in the NHE-1 inhibition. However, the complete reversal by LPA of the inhibitory effect of CNP on NHE supports that NPR-B in NPE cells are primarily involved in this response. On the other hand, the ability of LPA to enhance, when added alone, the NHE activity in NPE was unexpected, although not unique, because a similar effect has been described in tumor cells and smooth muscle cells (33, 38).

The present study also suggests that NPs, which are naturally occurring hormones locally synthesized within the ciliary body (15, 31), could modulate IOP through NHE-1 activity in
the CE. Although the present studies clearly demonstrate that NPs exhibited an inhibitory effect on the NHE-1 activity expressed in the NPE cells, we do not know how much the PE cell layer contributes to the overall activity or how the NHE activity is coregulated by these two cell layers. Additional work is needed before a comprehensive working model can be suggested to explain the potential interrelation between NP receptors in the tissues (CE and trabecular meshwork) of the anterior segment that govern IOP and NHE. However, recent findings may provide a possible physiological clue to the role of NHE in IOP (3). In these studies it has been shown that inhibitors of NHE-1 activity [i.e., dimethylamiloride (DMA), EIPA, and BIIB723] lower IOP when applied topically to the eye (3).

The colocalization of ANP and BNP along the NPE cell layer is consistent with the observation that they are secreted into aqueous humor. The level of NPs in aqueous humor is approximately sixfold higher than in plasma, BNP being the...
most abundant, followed by CNP and, at very low levels, ANP (14). The immunostaining of the vascular endothelium rather than the CE with CNP antibodies is consistent with the cell distribution of this NP in the endothelium of the blood vessels (43). Another site of action of NP released by the ciliary processes in the aqueous humor may be the trabecular meshwork and the Schlemm canal at the outflow system where NP receptors are present (7, 44).

It has been suggested that the ciliary bilayer functions as a syncytium (27). In this type of anatomic configuration, there is a possibility that the Na\(^{+}\)-dependent pH\(_i\) recovery recorded in NPE cells might reflect the sum of NHE activities in NPE and PE cell layers. One limitation of the present work is the inability to assess NHE activity directly at the PE cell side in the intact CE, and therefore the inability to determine whether PE cells contribute to the overall NHE-1 activity recorded on NPE cells. However, we attempted to address this question indirectly, by using uncoupling agents of intercellular junctions, including 18α-GA and heptanol. We observed that in the presence of CNP, 18α-GA was able to attenuate the inhibitory effect elicited by CNP when added alone on the rate of pH\(_i\) recovery. This effect suggested that the overall NHE-1 activity recorded in NPE cells may reflect the contribution of NHE-1 activity from PE cells as well, supporting the view that the NHE-1 activity in the CE is coregulated. Future studies using stroma-free preparations of CE will allow comparison of the NHE-1 activity of both PE and NPE cell sides of the bilayer.

The activity of NHE-1 was studied by pharmacological means using specific blockers such as EIPA and amiloride. The inhibition of ENaC and of the Na\(^{+}\)-K\(^{+}\)-2Cl\(^{-}\) cotransporter, respectively, did not alter the Na\(^{+}\)-dependent pH\(_i\) recovery of the NPE cells, suggesting that these transport systems are not likely to affect the NHE activity.

We think that an endocrine/paracrine cell mechanism could explain how NPs may influence NHE activity in the CE by creating a more hypertensive environment in the eye. CNP, the most potent of the three NPs in lowering IOP, presumably acts in a paracrine fashion on NPR-B receptors in the NPE-PE cells and presumably in cells of the outflow system. The activation of NPR-B receptors, leading to an increase in intracellular cGMP, will negatively affect the NHE in both NPE and PE cells, which could result in a decrease in ion and fluid transport into the aqueous humor. We believe that the source of NP in the aqueous humor is the CE for ANP and BNP and the vascular endothelium for CNP. The vascular endothelium of the ciliary processes may fulfill a storage function for CNP rather than being the site of synthesis. We think that ANP and BNP, on their secretion by the NPE cells, may target NPR-A or NPR-C by an autocrine mechanism in the same hormone-producing cells (NPE cells). Alternatively, ANP and BNP may target NPR-A in the paracrine vascular endothelial cells and enhance CNP release. Our observation that 8-Br-cGMP mimicked NP’s effect on NHE activity, as well as previous studies, suggests that CNP could activate NPR-B and presumably NPR-A in NPE cells, inducing an increase in intracellular cGMP production and thereby modulating NHE activity (28a, 44). Although cGMP-induced activation ofPKG appears to be the most likely intracellular mechanism of NHE inhibition, alternate pathways cannot be ruled out. CNP is known to exhibit a vasodilatory effect in the cardiovascular system and to exhibit a more lasting effect than BNP or ANP in lowering IOP in experimental animals. Levels of NP in the aqueous humor are likely to be modulated by the clearance activity of the NPR-C receptors in the NPE cells (31).

The present findings open up at least two potential ways in which a decrease in NHE activity could influence aqueous humor formation and possibly lower IOP: 1) NHE is involved in regulation of both cell volume and intracellular pH\(_i\), both of which have been identified as influencing transport activity across membranes, and 2) NPs likely influence other transport proteins through the second messenger cGMP. Which of these possible mechanisms, that is, a decrease in pH\(_i\), a change in cell volume, and/or an increase of intracellular cGMP levels, may have the greatest effect on aqueous humor formation and IOP will be the subject of further studies.

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