Presence of Functional Type B Natriuretic Peptide Receptor in Human Ocular Cells

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Purpose. To study the effects of natriuretic peptides on cyclic guanosine monophosphate (cGMP) production and calcium mobilization in cultured human ocular cells.

Methods. Cultured simian virus 40-transformed (HTM-3) and nontransformed (HTM-16) human trabecular meshwork (TM) cells and nontransformed human ciliary muscle (CM) cells were used. Accumulation of cGMP in cell lysate was measured by radioimmunoassay. Intracellular calcium concentration was measured by microscope-based ratiofluorometry.

Results. Both atrial natriuretic peptide (ANP) and C-type natriuretic peptide (CNP) increased the accumulation of cGMP in HTM-3, HTM-16, and CM cells. In the nontransformed TM cells, CNP was five times more efficacious (maximal effect of CNP was 497% ± 44% that of ANP) and 10 times more potent than ANP (ANP, log [EC50] = -6.99 ± 0.08; CNP, log [EC50] = -7.96 ± 0.20). Similar results were seen in HTM-3 and CM cells. Under the assay conditions used, the peptides increased only the production of cGMP without changing its degradation rate. The peptide-induced increase of cGMP in the TM and CM cells correlated with suppression of carbachol-induced calcium mobilization in the cell.

Conclusions. It is known that CNP, but not ANP, selectively activates the guanylyl cyclase associated with the type B natriuretic peptide receptor (NPR-B). Thus, the data suggest that NPR-B is the primary functional NPR in the TM and CM cells. The effects on cGMP and calcium produced by the activation of this receptor are expected to alter TM and CM contractility and may affect aqueous humor hydrodynamics and intraocular pressure. Invest Ophthalmol Vis Sci. 1996;37:1724-1731.
Type B Natriuretic Peptide Receptor

brain natriuretic peptide (BNP), whereas NPR-B is selective for the C-type natriuretic peptide (CNP) and, to a lesser degree, ANP and BNP. 12

Atrial natriuretic peptide was shown to increase cGMP production in ocular tissues and cells, such as the ciliary process 13,14 and CM cells. 9 This increase in cGMP predicts that ANP may lower IOP. Indeed, intracameral or intravitreal injection of ANP does lower IOP significantly in the rabbit. 15,16,17 It is unknown which NPR subtype mediates the ocular effects of ANP, even though a preliminary study reported that NPR-B was found in the cultured human TM cells. 17

For this article, we studied the effects of natriuretic peptides on cGMP production in the human TM and CM cells and, based on the pharmacologic profiles of these compounds, deduced the involvement of the specific NPR subtype. In addition, we investigated the functional consequences of activation of NPRs on intracellular calcium in the cultured human ocular cells.

METHODS

Three different cultured human ocular cell strains were used in this study: transformed trabecular meshwork cells (HTM-3 cells), nontransformed trabecular meshwork cells (HTM-16 cells), and nontransformed CM cells. The cultured human TM cell strain was a kind gift of Drs. Ernst Tamm and Elke Lütjen-Drecoll and was characterized as reported. 18 The nontransformed HTM-16 cells were obtained from a male donor, 18 years of age. Its isolation and characterization were described previously. 19 The transformed HTM-3 cells were transformed by an origin-defective simian virus 40 mutant. The details of its transformation and characterization were published previously. 19

Cells were cultured at 37°C and 5% CO 2 in Dulbecco’s modified Eagle’s medium (Gibco BRL, Grand Island, NY) with 10% fetal bovine serum (Hyclone, Logan, UT), supplemented with 4 mM L-glutamine (Gibco BRL) and 50 μg/ml gentamicin (Gibco BRL). HTM-3 and CM cells were subcultured after trypsinization, whereas HTM-16 cells were passaged using Cyto- dex 3 microcarrier beads (Sigma, St. Louis, MO). HTM-16 cells of passages 9 to 12, HTM-3 cells of passages 25 to 62, and CM cells of passages of 9 to 12 were used in the following experiments. All cell strains were free of mycoplasma contamination (assayed by MycoTect Mycoplasma Detection Kit; Gibco BRL).

Activity of guanylyl cyclase in these cells was estimated by the peptide-induced accumulation of cGMP as described. 20 Briefly, confluent cells in 48-well plates were incubated with serum-free medium containing 1 mM isobutylmethylxanthine (IBMX) at room temperature for 10 minutes (except in studies of Figure 2, where IBMX was withheld in some samples, as specified). Natriuretic peptides were then added and incubated for the indicated time period. The reaction was stopped, and cells were lysed by replacing the incubation medium with 0.2 ml of ice-cold 0.1 M acetic acid (pH 3.5). After 10 to 15 minutes, the acid was neutralized with 0.3 ml of 0.1 M sodium acetate (pH 12). An aliquot of the cell lysate was assayed for cGMP by radioimmunoassay (Biomedical Technologies, Stoughton, MA). The minimal detectable limit of cGMP by this assay was 10 fmol/0.5 ml cell lysate.

The intracellular calcium concentration ([Ca 2+] i) was assayed according to Shade et al. 21 Cells were cultured on sterilized #0 glass coverslips (Biophysics Technologies, Sparks, MD) for 3 to 7 days. On the day of study, the coverslip was placed into a mounting chamber (Medical Systems, Greenvyle, NY) and incubated at room temperature for 30 minutes with loading buffer (NaCl 125 mM, KCl 5 mM, CaCl2 1.8 mM, MgCl2 2 mM, NaH2PO4 0.5 mM, NaHCO3 5 mM, Hepes 10 mM, glucose 10 mM, bovine serum albumin 0.1%, fura-2 acetoxyethyl ester 5 μM, pH 7.2). After the incubation, the coverslip was rinsed twice with assay buffer (loading buffer without Fura-2 acetoxyethyl ester and bovine serum albumin) and mounted on the stage of a Nikon (Garden City, NY) Diaphot microscope. The chamber was filled with 2 ml of assay buffer at room temperature during the experiment. Test compounds were added in volumes of 20 μl and were removed by rinsing with 2 to 5 chamber volumes of assay buffer. Intracellular fluorescence intensity of 510 nm emission wavelength excited by alternating 340 and 380 nm excitation wavelengths was measured by a DeltaScan 4000 ratio fluorescence system (Photon Technology International, South Brunswick, NJ). The fluorescence intensity was monitored by a microscope photometer with photon-counting photomultiplier detector. The [Ca 2+] i was calculated from the intensity ratio of fluorescence at the two excitation wavelengths according to the equation of Grynkiewicz et al. 22

Human natriuretic peptides were obtained from Peninsula Laboratories (Belmont, CA). Carbachol and IBMX were purchased from Research Biochemicals (Natick, MA). Fura-2 acetoxyethyl ester was obtained from Molecular Probes (Eugene, OR). All other chemicals were obtained from Sigma.

RESULTS

Human Trabecular Meshwork Cells

In the cultured nontransformed human TM (HTM-16) cells, ANP, BNP, and CNP all stimulated the accumulation of cGMP in a concentration-dependent manner. The resting level of cGMP in lysates of these cells was 61.3 ± 34.1 fmol/well (mean ± SEM, n = 3).
Table 1). In contrast, CNP, the NPR-B receptor sub-
was similar to that of ANP, with a similar EC 50 value
Incubation of the cells with 10^(-9) M ANP for 15 minutes increased the accumulation of cGMP threefold to 171.3 ± 44.6 fmol/well (n = 3). The concentration-response curves were sigmoidal, with a mean calculated EC50 of 102.3 nM (log [EC50] = -6.99 ± 0.08, n = 3) (Fig. 1, Table 1). The stimulatory effect of BNP was similar to that of ANP, with a similar EC50 value (log [EC50] = -7.12 ± 0.04, n = 3) and similar maximal activity (1.85- ± 0.38-fold that of ANP) (Fig. 1, Table 1). In contrast, CNP, the NPR-B receptor subtype-selective agonist, was approximately 10 times more potent, with a mean EC50 of 11 nM (log [EC50] = -7.96 ± 0.20, n = 3) and was 4.97 ± 0.44 times more efficacious than ANP (Fig. 1, Table 1). These results demonstrate the presence of functional NPRs in the HTM-16 cells. Because CNP was significantly more potent and more efficacious than ANP and BNP and because the EC50 values of these peptides agree with those published for the NPR-B receptor subtype, these data further suggest that NPR-B was the prominent NPR in these cells.

Cultured human TM cells grow and proliferate extremely slowly. It is difficult to study them in assays that require significant numbers of cells. The faster growth rate and relatively long-term stability of transformed cells can provide an acceptable and useful alternative for many biochemical and pharmacologic studies. This is especially relevant because immortalization of TM cells by transformation with origin-defective SV40 was previously shown not to affect most receptor-signal transduction pathways and pharmacologic responses of the cell. Therefore, we tested the HTM-3 cells for their responsiveness to ANP, BNP, and CNP. Indeed, the natriuretic peptides were also effective in increasing cGMP accumulation in the transformed cells. In these cells, the basal level of cGMP was 18.6 ± 13.1 fmol/well (n = 6). Treatment of the cells with 10 μM ANP increased the cGMP level to 150 ± 47 fmol/well (n = 5). The action of ANP was concentration dependent, with a mean calculated EC50 of 479 nM (log [EC50] = -6.32 ± 0.06, n = 4) (Fig. 1, Table 1). Brain natriuretic peptide (log [EC50] = -6.40 ± 0.17, n = 4) was equipotent to ANP, even though its maximal effect was 4.32 ± 0.58 (n = 4) times that of ANP (Fig. 1, Table 1). Again, in contrast with ANP and BNP and similar to its effect in nontransformed TM cells, CNP, the NPR-B selective agonist in HTM-3 cells, was an order of magnitude more potent than ANP (log [EC50] = -7.19 ± 0.16, n = 4) and 33.47 ± 2.44 (n = 3) times more efficacious (Fig. 1, Table 1). The pharmacologic profiles of these compounds imply that, comparable to the nontransformed cells, the NPR-B was also the prominent subtype of NPR in the HTM-3 cells; they suggest additionally that transformation did not significantly affect the expression of the receptor in TM cells.

The stimulation of natriuretic peptides on cGMP accumulation in the HTM-3 cells was caused by their activation of guanylyl cyclase. In the assay conditions used, the effect of the peptides on the degradation rate of cGMP should be negligible. The hydrolysis of cGMP was already completely blocked by the inclusion of IBMX, a nonselective phosphodiesterase inhibitor, in the assay buffer. As evidenced in Figure 2, the decline of cGMP was profound without IBMX. Most cGMP (75% to 90%, n = 3) was destroyed within a 10-minute incubation period. The addition of 1 mM IBMX completely inhibited such decline. Because cells in all other studies were preincubated with IBMX, the increase in cGMP accumulation by the peptides cannot be explained by an inhibition of the degradation pathway. Furthermore, time-course studies indicate that the difference between ANP and CNP was
TABLE 1. Effect of Natriuretic Peptides on Cyclic Guanosine Monophosphate Production in Human Ocular Cells

<table>
<thead>
<tr>
<th></th>
<th>TM-16 Cells</th>
<th>TM-3 Cells</th>
<th>CM Cells</th>
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<tbody>
<tr>
<td>ANP</td>
<td></td>
<td></td>
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<tr>
<td>log [EC&lt;sub&gt;50&lt;/sub&gt;]</td>
<td>-6.99 ± 0.08 (n = 3)</td>
<td>-6.32 ± 0.06 (n = 4)</td>
<td>-6.99 ± 0.18 (n = 6)</td>
</tr>
<tr>
<td>Max effect (%)</td>
<td>100 (n = 3)</td>
<td>100 (n = 4)</td>
<td>100 (n = 6)</td>
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<tr>
<td>BNP</td>
<td></td>
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<tr>
<td>log [EC&lt;sub&gt;50&lt;/sub&gt;]</td>
<td>-7.12 ± 0.04 (n = 3)</td>
<td>-6.40 ± 0.17 (n = 4)</td>
<td>-7.45 ± 0.19 (n = 3)</td>
</tr>
<tr>
<td>Max effect (%)</td>
<td>185 ± 38 (n = 3)</td>
<td>432 ± 58 (n = 4)</td>
<td>115 ± 33 (n = 3)</td>
</tr>
<tr>
<td>CNP</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>log [EC&lt;sub&gt;50&lt;/sub&gt;]</td>
<td>-7.96 ± 0.20 (n = 3)</td>
<td>-7.19 ± 0.16 (n = 4)</td>
<td>-7.70 ± 0.10 (n = 6)</td>
</tr>
<tr>
<td>Max effect (%)</td>
<td>497 ± 44 (n = 3)</td>
<td>3347 ± 244 (n = 3)</td>
<td>520 ± 107 (n = 6)</td>
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Values are mean ± SEM. Max effect = cyclic guanosine monophosphate level at 10 μM of the indicated peptide compared with the maximal effect of 10 μM ANP, which defines 100%.

TM = trabecular meshwork; CM = ciliary muscle; ANP = atrial natriuretic peptide; BNP = brain natriuretic peptide; CNP = C-type natriuretic peptide.

their effect on the production rate of cGMP. A representative study is shown in Figure 3, in which the initial production rates of cGMP after CNP (30 nM or 1 μM) treatment were significantly higher than those after ANP (1 μM) treatment. The direct stimulating effects of ANP, BNP, and CNP on the production of cGMP confirm that the NPR guanylyl cyclase was involved. The selectivity of this effect toward CNP substantiates the involvement of the NPR-B subtype.

Cyclic GMP causes relaxation of various smooth muscles, presumably by lowering [Ca<sup>2+</sup>]. In the nontransformed human TM (HTM-16) cells, we demonstrated previously that carbachol, through the M3

FIGURE 2. Effect of the phosphodiesterase inhibitor, isobutylmethylxanthine (IBMX), on the degradation of cyclic guanosine monophosphate (cGMP) in the HTM-3 cells. Cells were treated with C-type natriuretic peptide (1 μM) in the absence of IBMX for 15 minutes, and then IBMX (1 mM) or vehicle was added. Cyclic GMP was assayed at the indicated period after IBMX treatment. Each symbol represents a single datum point. Similar data were obtained in three independent studies, each in duplicates.

FIGURE 3. Time courses of increase in cyclic guanosine monophosphate (cGMP) induced by natriuretic peptides in HTM-3 cells. Cyclic GMP concentration was measured after the cells were treated with natriuretic peptides for the indicated period. Each symbol represents a single datum point. The lower panel shows the atrial natriuretic peptide effect with magnified y-axis. Similar data were obtained in three independent studies, each in duplicate.
FIGURE 4. Effect of natriuretic peptides on carbachol-activated calcium mobilization in nontransformed human trabecular meshwork (TM) cells. (upper panel) Calcium response of a single cell. HTM-16 cells were treated with carbachol (CCh, 100 μM), C-type natriuretic peptide (CNP; 100 nM), or atrial natriuretic peptide (ANP; 100 nM) as indicated. “Wash” represents removal of drugs by rinsing, during which data collection was suspended for 2 to 3 minutes. Similar results were obtained in six studies. (lower panel) Summary data indicating the suppression of carbachol-induced calcium mobilization by ANP and CNP (presented as mean percentage inhibition ± SEM). Statistical significance between the two groups was obtained by two-tailed Student's t-test.

Human Ciliary Muscle Cells

Similar to its effect in the TM cells, CNP, in the CM cells, was more potent and more efficacious in stimulating the production of cGMP than ANP or BNP (Fig. 5, Table 1). The basal cGMP level in the CM cells was 121.5 ± 33.1 fmol/well (n = 6). ANP produced a concentration-dependent increase in cGMP with a maximal cGMP level of 282.5 ± 57.1 fmol/well (n = 6) and a mean calculated EC50 value of 102.7 nM (log [EC50] = -6.99 ± 0.18, n = 6). BNP was slightly more potent than ANP (log [EC50] = -7.45 ± 0.19, n = 3) with similar efficacy: maximal effect of BNP was 1.15–± 0.33-fold (n = 3) that of ANP. CNP was 5.20–± 1.07-fold (n = 6) more efficacious than ANP and almost 10-fold more potent (log [EC50] = -7.70 ± 0.10, n = 6) (Fig. 5, Table 1). The potencies and efficacies of the natriuretic peptides suggest that the same NPR-B is the primary functional NPR in the cultured human CM cells.

In the CM cells, carbachol was effective in activating intracellular calcium mobilization with a peak phase and a sustained plateau.25 When added at the plateau, CNP (100 nM) produced effects similar to those in the TM cells. It partially reversed the carbachol (100 μM)-activated calcium mobilization (Fig. 6). Even though its effect was small (mean inhibition =

FIGURE 5. Effect of natriuretic peptides on cyclic guanosine monophosphate level in human ciliary muscle cells. Cyclic guanosine monophosphate concentration was measured after the cells were treated with natriuretic peptides for 15 minutes. Each symbol represents a single datum point. Similar results were obtained in six independent studies, each in duplicate.
Type B Natriuretic Peptide Receptor

FIGURE 6. Effect of C-type natriuretic peptide (CNP) on carbachol-activated calcium mobilization in human ciliary muscle cells. Cells were treated with carbachol (CCh, 100 μM) and CNP (100 nM) as indicated. “Wash” represents removal of drugs by rinsing, during which data collection was suspended for 2 to 3 minutes. Similar results were obtained in five studies.

21% ± 2%, n = 6), it was reproducible. Contrarily, the effect of 100 nM ANP was inconsistent and, in most cases, difficult to detect (data not shown).

DISCUSSION

In this article, we have demonstrated that in human TM cells, natriuretic peptides, such as ANP, BNP, and CNP, were effective in increasing the accumulation of cGMP through the activation of guanylyl cyclase. Among the peptides tested, CNP was the most potent and efficacious. The potency profile, CNP > ANP = BNP, and respective EC50 values indicate that NPR-B is the prominent functional receptor subtype in these cells. Our findings agree with results presented in a preliminary report by Lehman et al.,17 in which CNP was found to be more potent than ANP in competing with the binding of radioactive ANP and in increasing cGMP accumulation in human TM cells. We further showed that, in addition to the TM cells, human CM cells displayed the same selective responses toward CNP and that the NPR-B is the likely functional receptor subtype in the CM cells as well. We speculate that the presence of NPR-B in the TM and CM may contribute to the modulation of aqueous outflow facility.

The presence of NPR-B in ocular cells is apparently not universal. Not all human ocular tissues or cells have NPR-B. CNP and ANP were approximately equal in potencies and efficacies in increasing the accumulation of cGMP (maximal cGMP level less than three times the basal level) in transformed human nonpigmented ciliary epithelial cells (Pang IH, unpublished observation, 1995), suggesting the presence of the NPR-A receptor subtype in these cells instead. The significance of the NPR receptor in the ciliary epithelial cells is unclear, but natriuretic peptides are known to affect fluid and ion transport. Carre and Civan78 showed that cGMP can modulate ion transport across the ciliary epithelium. Hence, the activation of NPRs in this tissue may affect the production of aqueous humor.

We excluded the likelihood that stimulation of cGMP accumulation by natriuretic peptides was caused by their actions on the degradation of cGMP. Under the conditions used, in which a phosphodiesterase inhibitor, IBMX, was included routinely in the samples, degradation of cGMP was nonexistent. Consequently, our data strongly indicate that increases of cGMP in cell lysates were caused by increases in its production. This was further corroborated by the time course study in which the initial rate of cGMP production was many times higher for CNP than for ANP, confirming that the activation of the natriuretic peptide receptor, guanylyl cyclase, was the primary cause of cGMP accumulation. Interestingly, the amount of cGMP reached a plateau at approximately 15 minutes after the treatment of either ANP or CNP, and evidently little cGMP was produced after that. The reason for this phenomenon is unknown. It may indicate rapid desensitization of the receptor or perhaps significant hydrolysis of the peptides by cell-associated peptidase(s).

In these studies, we also used transformed TM cells. The responses between the transformed and nontransformed TM cells to the peptides were similar but not identical. For example, the EC50 values of the agonists were consistently approximately 0.7 to 0.8 log unit less potent in the HTM-3 cells. The maximal effect of CNP on the HTM-3 cells was approximately 33 times that of ANP, compared to five times that of ANP in the HTM-16 cells. Nevertheless, the effect of CNP was always significantly more potent and more efficacious than ANP or BNP in both cell strains. These findings suggest that transformation of the TM cells did not affect the expression of NPR-B as the prominent functional receptor subtype. However, it probably has influenced the total receptor number or its density on the cell.

Cyclic GMP has been shown to cause relaxation of many smooth muscle types. Its effect may involve many mechanisms, one of which is the decrease of intracellular concentration of free calcium. The guanylyl cyclase-stimulating effect of natriuretic peptides suggests their potential action on calcium levels in the cell. We demonstrated in this study that CNP did not affect resting cell calcium concentration but lowered carbachol-activated calcium mobilization in HTM-16 and CM cells. Its effect was invariably more efficacious.
than ANP, reflecting the difference in their potencies for cGMP production. The mechanism of this calcium-
lowering effect is unclear. However, cGMP is known to lower [Ca^{2+}], by stimulation of the cGMP-dependent
protein kinase,\textsuperscript{20} increase of the Na^{+}–Ca^{2+} exchange,\textsuperscript{39} and enhancement of calcium sequestration
by activation of the sarcoslemmal Ca^{2+} pump,\textsuperscript{31,32} the plasmalemmal Ca^{2+} pump, or both.\textsuperscript{33} It will be inter-
esting to discover which of the above mechanisms is involved in the human ocular tissues and cells and
whether TM and CM use the same or different mecha-
nisms. Although the addition of CNP to carbachol-
treated cells lowered [Ca^{2+}], the pretreatment of cells
with CNP did not diminish calcium mobilization by the subsequent addition of carbachol. Perhaps rapid
desensitization of the receptor or swift degradation of the peptide was responsible.

The [Ca^{2+}]-lowering and guanylyl cyclase-stimu-
lating effects of CNP probably add relaxation of the TM and CM tissues because activation of NPRs was shown to relax other smooth muscles, such as smooth muscles of the vasculature.\textsuperscript{54–56} More important, cGMP analogues and nitrovasodilators were shown to relax both precontracted CM and TM tissue strips.\textsuperscript{7,8} Relaxation of these ocular structures, which are known to be involved in the regulation of aqueous outflow facility, should cause changes in IOP. Our findings on pharmacologic profiles of the natriuretic peptides suggest that NPR-B agonists, such as CNP, will be more potent and effective in these actions. Indeed, Takashima et al\textsuperscript{79} reported that natriuretic peptides lower IOP in the rabbit eye. In their studies, CNP was more effective than ANP or BNP. In addition to the muscle-relaxing effects, the activation of natriuretic peptide receptors influences many other cell functions, such as inhibition of renin release, aldosterone synthesis, evoked-catecholamine synthesis, angiotensin converting enzyme activity, reflex sympathetic neuronal activity, and adrenocorticotropic hormone release (see review by Drewett and Garbers\textsuperscript{11}). Whether these effects alter aqueous hydrodynamics or other ocular functions remains to be determined. In summary, we have demonstrated that NPR-B is the primary functional NPR in human TM and CM cells. Activation of this receptor is likely to affect the aqueous hydrodynamics.

**Key Words**
ciliary muscle, cyclic guanosine monophosphate, natriuretic peptide, NPR-B, trabecular meshwork

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**References**

18. Tamm E, Flügel C, Baur A, Lütjen-Drecoll E. Cell


