Characterization of Natriuretic Peptide Receptors in Cultured Cells
Shin-ichi Suga, Kazuwa Nakao, Masashi Mukoyama, Hiroshi Arai, Kiminori Hosoda, Yoshihiro Ogawa, and Hiroo Imura

To elucidate physiological and clinical implications of the natriuretic peptide family, the expression of receptors for natriuretic peptides has been examined in cultured cells (a rat pheochromocytoma cell line [PC12], bovine endothelial cells, rat aortic smooth muscle cells, human mesangial cells, and a porcine kidney epithelial cell line [LLC-PK1]) by Northern blot analysis and cyclic GMP production method for the ANP-A and ANP-B receptors and by Northern blot analysis and binding assay for the clearance receptor. The ANP-A receptor was predominantly expressed in PC12 cells, bovine endothelial cells, and LLC-PK1 cells but was barely expressed in rat aortic smooth muscle cells and human mesangial cells. By contrast, the ANP-B receptor was the major subtype of the biologically active receptors in rat aortic smooth muscle cells and human mesangial cells. Only a small amount of the ANP-B receptor was detected in PC12 cells, bovine endothelial cells, and LLC-PK1 cells. The clearance receptor was abundantly expressed in rat aortic smooth muscle cells and human mesangial cells and was also present in bovine endothelial cells, but it was undetectable in PC12 cells and LLC-PK1 cells. These results demonstrate that the expression of three natriuretic peptide receptors varies from cell to cell, which is relevant to cell- or tissue-specific action of the natriuretic peptide family. (Hypertension 1992;19:762–765)

KEY WORDS • natriuretic peptides • pheochromocytoma • endothelium • vascular smooth muscle cells • mesangial cells • renal epithelial cells

Atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), and C-type natriuretic peptide (CNP) have been considered to comprise the natriuretic peptide family responsible for body fluid homeostasis and blood pressure control.1-9 Receptors for natriuretic peptides have been shown to exist in various target tissues and cells.5,10-13 Two distinct types of receptors have been identified. One type of receptor is a particulate guanylate cyclase14 and is designated as the biologically active receptor. Molecular cloning has revealed the existence of two subtypes of biologically active receptors, the ANP-A receptor and ANP-B receptor.15 The other type of receptor is not coupled to guanylate cyclase16 and is proposed to have a major role in the clearance of natriuretic peptides.10 This receptor is, therefore, termed the clearance receptor (C receptor).

Recently, we and others have demonstrated that ANP is a selective ligand for the ANP-A receptor, with the rank order of potency for cyclic GMP (cGMP) production $\text{ANP} \geq \text{BNP} \geq \text{CNP}$, and that CNP is a selective ligand for the ANP-B receptor, with the rank order of potency $\text{CNP} \geq \text{ANP} \geq \text{BNP}$.12,17 We have also revealed the C receptor selectivity with the rank order of the binding affinity, $\text{ANP} > \text{CNP} > \text{BNP}$.12 Knowledge of the expression of three receptors for natriuretic peptides at the cellular level have, therefore, important functional consequence.

In the present study, to elucidate the physiological and clinical implications of the natriuretic peptide family, the expression of the three kinds of natriuretic peptide receptors was examined in various cultured cells with the binding assay, cGMP production method, and Northern blot analysis.

Methods

Cell Culture

A rat pheochromocytoma cell line (PC12), rat aortic smooth muscle cells (SMC), bovine endothelial cells (EC), and human mesangial cells were cultured as we reported.12 A porcine kidney epithelial cell line (LLC-PK1) was cultured as described.11

Analysis of C Receptor by Binding Assay

The expression of the C receptor was examined by the competitive binding study of $^{125}$I-rat atrial natriuretic peptide (ANP)-(99–126) by human or rat ANP-(99–126) (Peptide Institute Inc., Minoh, Japan) and a selective ligand for the C receptor, des[Glu$^9$,Ser$^9$,Gly$^{10}$,Leu$^{11}$,Gly$^{12}$]ANP-(4–23)-NH$_2$ [C-ANP-(4–23)] (donated by Professor T. Maack, Cornell University Medical College, Ithaca, N.Y.),10 as previously reported.12 Briefly, cells were incubated at 4°C for 2 hours with $^{125}$I-rat ANP-(99–126) (200–400 pM) and the competing ligand in 250 μl Hanks’ balanced salt solution. Nonspecific binding was determined using 1 μM unlabeled ANP-(99–126).

Analysis of ANP-A and ANP-B Receptors by Cyclic GMP Production

The expression of the ANP-A and ANP-B receptors was determined by the effect of ANP and CNP on...
cGMP production in cultured cells, on the basis of the observation that ANP is at least a thousandfold stronger than CNP in cGMP production via the ANP-A receptor and that CNP is more potent than ANP in cGMP production via the ANP-B receptor. The cells were incubated at 37°C for 30 minutes in 500 μl Dulbecco’s modified Eagle’s medium containing 0.1% bovine serum albumin and 0.5 mM isobutylmethylxanthine (Sigma Chemical Co., St. Louis, Mo.) with ANP-(99–126) or CNP (donated by Dr. K. Inouye, Shionogi Laboratories, Shionogi & Co., Ltd., Osaka, Japan). The amount of cGMP was determined by radioimmunoassay as we previously reported.

RNA Extraction and Northern Blot Hybridization

Total RNA was extracted from cultured cells as described. Rat ANP-A receptor and rat ANP-B receptor complementary DNA (cDNA) probes were prepared as we reported. Since cDNA of rat C receptor has not been cloned, bovine C receptor cDNA probe, corresponding to nucleotides 1423-2070 of bovine C receptor, was prepared by cDNA synthesis and the polymerase chain reaction (PCR) method. These three fragments were labeled by the random-primed method (specific activity approximately 1x10^6 cpm • μg^-1). Northern blot analyses of ANP-A, ANP-B, and C receptor messenger RNA (mRNA) were performed, using 5 μg of total RNA as we reported.

Data Analysis

Results are presented as the means of three separate experiments. The SEM was less than 10% of the mean.

Expression of C Receptor

Competitive inhibition of [125I]atrial natriuretic peptide (ANP)-(99–126) binding by C-ANP-(4–23) in PC12 cells (Figure 1A) revealed that C-ANP-(4–23) failed to compete for the ANP binding sites even at extremely high concentration (1 μM). A similar result was obtained in LLC-PK₁ cells. Therefore, the overwhelming majority of the receptors expressed in PC12 cells and LLC-PK₁ cells is classified as the biologically active receptor. In the binding assay with bovine EC (Figure 1B), C-ANP-(4–23) displaced [125I]ANP-(99–126) from approximately 20% of the ANP binding sites with a high affinity; however, it failed to displace [125I]ANP-(99–126) from the remaining binding sites. In marked contrast, in rat aortic SMC (Figure 1C) and human mesangial cells, C-ANP-(4–23) competed for 96% and 92% of the total ANP binding sites with high affinities, respectively, indicating that the majority of the receptors in rat aortic SMC and human mesangial cells are the C receptor. The total number of natriuretic peptide receptors was 75,000 sites/cell in PC12 cells, 21,000 sites/cell in bovine EC, 66,000 sites/cell in LLC-PK₁ cells, 320,000 sites/cell in rat aortic SMC, and 22,000 sites/cell in human mesangial cells.

Expression of ANP-A and ANP-B Receptors in Cultured Cells

Figure 2 shows the effects of ANP-(99–126) and CNP on cGMP production in cultured cells. In PC12 cells (Figure 2A), bovine EC (Figure 2B), and LLC-PK₁ cells (Figure 2C), ANP-(99–126) stimulated the cGMP production at 100 nM, and concentrations of ANP necessary to stimulate half-maximal production of cGMP (EC₅₀) were approximately 2.9, 1.7, and 2.8 nM, respectively. In contrast, CNP had little effect on cGMP production below 100 nM and was at least 300-fold less potent than ANP-(99–126) in these three types of cells. These findings are consistent with the previous observations on cGMP production via the ANP-A receptor. In rat aortic SMC (Figure 2D) and human mesangial cells (Figure 2E), CNP was more potent than ANP-(99–126) in cGMP production. However, EC₅₀ of CNP in rat aortic SMC and human mesangial cells was at least one order of magnitude higher than that of ANP-(99–126) in PC12 cells, bovine EC, and LLC-PK₁ cells. These results are also identical to the previous findings on cGMP production via the ANP-B receptor.

Northern Blot Analysis

ANP-A receptor, ANP-B receptor, and C receptor mRNA levels were measured by Northern blot analysis with specific cDNA probes. Figure 3 shows results of Northern blotting in PC12 cells, rat aortic SMC, and bovine EC. RNA extracted from PC12 cells contained an intense hybridizing signal of ANP-A receptor mRNA of approximately 4.0 kb, and ANP-B receptor mRNA of the same size was present in a limited quantity. However, C receptor mRNA was undetectable when 5 μg total RNA was used. A similar pattern was observed in LLC-PK₁ cells, but the level of the ANP-B receptor mRNA was slightly lower than that in PC12 cells. By contrast, in rat aortic SMC and human mesangial cells,
FIGURE 2. Line plots show cyclic GMP (cGMP) production by atrial natriuretic peptide (○) and C-type natriuretic peptide (●) in PC12 cells (panel A), bovine endothelial cells (panel B), LLC-PK, cells (panel C), rat aortic smooth muscle cells (panel D), and human mesangial cells (panel E).

ANP-A receptor mRNA was not detectable, but ANP-B receptor and C receptor mRNA were abundantly expressed. RNA extracted from bovine EC contained intense hybridizing signals of ANP-A receptor mRNA and C receptor mRNA, but it contained only a weak hybridizing signal of ANP-B receptor mRNA. In addition, multiple hybridizing bands of C receptor mRNA were present in rat aortic SMC and bovine EC, being consistent with previous observations.


discussion
In the present study, using the binding assay, cGMP production method, and Northern blot analysis, the expression of natriuretic peptide receptors in five types of cultured cells was characterized, demonstrating that the expression of the three receptors for natriuretic peptides varies from cell to cell. Table 1 summarizes the results of characterization of natriuretic peptide receptors in cultured cells. Most of the receptors expressed in rat aortic SMC and human mesangial cells are the C receptor, whereas the biologically active receptor is the major receptor in PC12 cells, LLC-PK, cells, and bovine EC. As for the subtype of the biologically active receptors, the ANP-A receptor is predominantly expressed in PC12 cells, bovine EC, and LLC-PK, cells, whereas the ANP-B receptor is predominant in rat aortic SMC and human mesangial cells.

Since CNP is distributed mainly in the brain, the significance of the abundant expression of the ANP-B receptor in mesangial cells and aortic SMC remains to be resolved. However, we have reported that CNP exists in peripheral organs, such as in pituitary, kidney, and colon. Therefore, CNP may act as a local regulator through interaction with the ANP-B receptor.

In conclusion, the present study demonstrates the cell-specific expression of three receptors for natriuretic peptides. It is not clear, at present, whether receptors expressed in cultured cells really reflect the in vivo pattern of expression; however, the cell-specific expression of the natriuretic peptide receptors is relevant to the cell- or tissue-specific actions of the natriuretic peptide family.

**TABLE 1. Expression of Natriuretic Peptide Receptors in Cultured Cells**

<table>
<thead>
<tr>
<th>Cultured cells</th>
<th>ANP-A receptor</th>
<th>ANP-B receptor</th>
<th>C receptor</th>
</tr>
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<tbody>
<tr>
<td>PC12 cells</td>
<td>+++</td>
<td>+</td>
<td>− ± ± ± ±</td>
</tr>
<tr>
<td>LLC-PK, cells</td>
<td>+++</td>
<td>±</td>
<td>− ± ± ± ±</td>
</tr>
<tr>
<td>Bovine endothelial cells</td>
<td>+++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Rat aortic SMC</td>
<td>− ±</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Human mesangial cells</td>
<td>− ± ± ± + ++</td>
<td>++</td>
<td>++</td>
</tr>
</tbody>
</table>

ANP, atrial natriuretic peptide; C receptor, clearance receptor; SMC, smooth muscle cells.

**FIGURE 3. Northern blot analysis of ANP-A receptor, ANP-B receptor, and clearance receptor (C receptor) messenger RNA in PC12 cells, rat aortic smooth muscle cells (RASMC), and bovine endothelial cells (BEC).** Five micrograms total RNA were used for analysis. ANP, atrial natriuretic peptide.
tors at the cellular level will be of great help to solve the problem.

References

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