ATRIAL NATRIURETIC PEPTIDE BINDING SITES ON HOG CILIARY BODIES AND CHOROID

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ABSTRACT

Binding studies revealed the presence of two classes of binding sites for atrial natriuretic peptide (ANP) on hog ciliary bodies and choroid membranes. The curvilinear Scatchard plots were evaluated by nonlinear regression analysis using a two-site model which identified a high affinity site $K_d1 = 73 \pm 12 \text{ pM}$ and $B_m1 = 77 \pm 7 \text{ fmol/mg protein}$, and a low affinity site $K_d2 = 0.748 \pm 0.199 \text{ nM}$ and $B_m2 = 44 \pm 11 \text{ fmol/mg protein}$ in ciliary bodies, and a high affinity site $K_d1 = 45 \pm 3 \text{ pM}$ and $B_m1 = 44 \pm 2 \text{ fmol/mg protein}$, and a low affinity site $K_d2 = 1.18 \pm 0.356 \text{ nM}$ and $B_m2 = 158 \pm 44 \text{ fmol/mg protein}$ in choroid. Affinity labeling with disuccinimidyl suberate followed by electrophoresis under nonreducing condition and autoradiography show that the peptide binds predominantly to 66,000 dalton species on membrane of ciliary bodies. However, on membrane of choroid, the high molecular size protein (130,000 dalton species) is intensely labeled. The results suggest that a polypeptide of 66,000 dalton represents a component of the high affinity ANP receptor.

Atrial natriuretic peptides (ANPs), recently discovered cardiac hormone, have been shown to elicit diuresis, natriuresis, vasorelaxant activities and to inhibit secretion of aldosterone (1, 4, 5, 13). Specific binding sites for ANPs have been identified in the kidney (10, 21), adrenal gland (6, 15), brain (12), placenta (16), ovary (S.-J. Kim, M. Shinjo, S. Usuki, H. Miyazaki, M. Tada and K. Murakami, Binding sites for atrial natriuretic peptide in high concentrations in human ovary; in preparation), lung (11), liver (20), and vascular system (10, 15). However, there is little information about the molecular properties of the ANP receptors in these tissues. Previous reports have demonstrated that epithelial cells in ciliary bodies have binding sites for $^{125}$I-ANP, indicating that this peptide is involved in the regulation of water and/or electrolyte transport in this tissue (2). The choroid, which is a highly vascular tissue between the retina and the sclera may be involved in the regulation of aqueous humor flow and intraocular pressure.

In this communication, we examined the specific binding sites for ANP in hog ciliary bodies and choroid in an attempt to provide information about the presence and distribution of ANP binding sites in the eye.

MATERIALS AND METHODS

Materials

$^{125}$I-rat ANP ($^{125}$I-rANP, 2,087 Ci/mmol) and $^{14}$Cmethylated protein markers were purchased from Amersham, U.K.; synthetic rat ANP from Peptide Institute, Osaka, Japan; disuccinimidyl suberate (DSS) from Pierce,
U.S.A.; leupeptin and antipain from Protein Research Foundation, Osaka, Japan; X-ray film (RX-50) and developing solutions from Fuji Film, Japan.

Tissue and Membrane Preparations
Fresh hog eyes were obtained from a local slaughterhouse. The enucleated eyes were dissected into ciliary bodies and choroid. Eye tissues were homogenized in a 50 mM Tris-HCl (pH 7.1) containing 5 mM EDTA and 2 µg/ml each of leupeptin and antipain by 5.5 strokes on a Polytron PT 10/35. The homogenates were centrifuged at 10,000 g for 10 min at 4°C and the supernatants were centrifuged at 105,000 g for 45 min at 4°C. The pellets were again homogenized in phosphate-buffered saline (PBS: 10 mM phosphate, pH 7.4, containing 120 mM NaCl and 10 mM EDTA) and recentrifuged as before. The washed pellets were stored at −80°C until use. 

125I-rANP Binding Assay
The frozen membrane preparations were thawed and suspended at 0.3–0.5 mg of protein/ml in PBS containing 20 µg/ml each of leupeptin and antipain. Aliquots of 180 µl of diluted membrane suspensions were incubated with 10,000 cpm of 125I-rANP (10 pM) in the total volume of 200 µl containing 10 µl of unlabeled ANP of various concentrations. After incubation for 30 min at 20°C, 1 ml of cold PBS containing 0.1% bovine serum albumin was added and filtered through Whatman GF/F filter. Filters were washed two times with an additional 2 ml of the same buffer. Filters were assayed for radioactivity in a Beckman γ-counter. The biphasic Scatchard plots were analyzed by nonlinear regression analysis.

Affinity Labeling, Electrophoresis, and Autoradiography
Paired tubes containing 50 µl of 125I-rANP (3×10² cpm) and 500 µl of membrane suspension (5–10 mg membranes in PBS containing 20 µg/ml each of leupeptin and antipain) were incubated in the presence or absence of 500 nM unlabeled ANP at 20°C for 30 min. The bound 125I-rANP was covalently cross-linked by adding 0.5 mM DSS. Following a 10-min incubation at 20°C, the cross-linking reaction was terminated by adding 30 µl of 2 M ammonium acetate, and membranes were recovered and washed by centrifugation at 20,000 g for 10 min. Covalently labeled receptors were then analysed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was performed under reducing and nonreducing conditions on gradient slab gel, prepared by mixing equal volumes of 13.5% and 3% acrylamide solutions with other procedure as in the method of Laemmli (9). After electrophoresis, the gels were dried and exposed to X-ray film for 25 days at −70°C with enhancing screen.

RESULTS
The bindings of 125I-rANP to hog ciliary bodies and choroid membrane preparations were specific and constituted more than 60%

[Graph: Binding kinetics of 125I-rANP to hog ciliary bodies (—) and choroid (—) membranes. The radiolabeled peptide was incubated at a concentration of 10 pM. Specific binding was defined as total binding minus nonspecific binding in the presence of 1.8 µM unlabeled ANP. Each point is the mean of two determinations.]
to quantitate two classes of binding sites by the use of nonlinear regression analysis. Some variabilities in the number and affinity were observed from two separate membrane preparations. The results from membrane preparations of hog ciliary bodies and choroid were summarized in Table 1. In ciliary bodies, value of \( B_{\text{max}} \) of high affinity binding sites (\( B_{\text{max}1} \)) was higher than that of low affinity binding sites (\( B_{\text{max}2} \)) (77±7 and 44±11 fmol per mg protein for high and low affinity binding sites, respectively). However, value of \( B_{\text{max}1} \) in choroid was lower than that of \( B_{\text{max}2} \) (44±2 and 158±44 fmol per mg protein for high and low affinity binding sites, respectively). Comparison of the total number of receptor sites for ANP on membrane preparations of ciliary bodies and choroid showed that choroid has more binding sites than ciliary bodies. However, the number of ANP receptor sites with high affinity is greater in ciliary bodies than in choroid.

The putative membrane surface receptors for ANP in both ciliary bodies and choroid have been identified with a bifunctional cross-linking reagent (DSS). To determine if ciliary bodies and choroid possess multiple binding sites for ANP as suggested by the equilibrium binding studies (Fig. 2 and Table 1), membrane preparations of ciliary bodies or choroid were cross-linked with \( 125\text{I}-\text{rANP} \) in the presence or absence of excess unlabeled ANP, and the proteins were then separated by SDSPAGE. The ANP binding proteins in both tissues appear identical, consisting of 66,000 and 130,000 \( M_r \) proteins, despite the differences in \( K_d \) and \( B_{\text{max}} \) for the peptide. The predominant binding sites in the ciliary bodies had a molecular size of approximately 66,000 daltons, whereas the second site faintly labeled had a molecular size of approximately 130,000 daltons (Fig. 3A). The relative proportion of this

![Graph](https://via.placeholder.com/150)

Fig. 2  Scatchard analysis of \( 125\text{I}-\text{rANP} \) binding to hog ciliary bodies and choroid membranes. Membrane suspensions were incubated with \( 125\text{I}-\text{rANP} \) in the presence of increasing amounts of unlabeled rANP and the quantity of bound radioactive ligand was determined as described under Materials and Methods. Closed circles (---) show the \( 125\text{I}-\text{rANP} \) binding curve of ciliary bodies membrane, and open circles (---) show the \( 125\text{I}-\text{rANP} \) binding curves of choroid membrane.

of the total binding. Specific binding of \( 125\text{I}-\text{rANP} \) was observed when 10 pM labeled ligand was incubated with membrane preparations of ciliary bodies or choroid. The bindings of \( 125\text{I}-\text{rANP} \) to these preparations at 22°C were rapid, and reached the maximum levels in 15–30 min (Fig. 1).

On Scatchard analysis of the binding isotherms, apparent biphasic curves were obtained (Fig. 2). These data have been analysed

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### Table 1  Equilibrium Binding Parameters for ANP of Porcine Ciliary Bodies and Choroid Membranes

<table>
<thead>
<tr>
<th>Tissues</th>
<th>Dissociation constants</th>
<th>Binding sites</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>( K_d^1 ) (nM)</td>
<td>( B_{\text{max}}^1 ) (fmol/mg protein)</td>
</tr>
<tr>
<td>Ciliary bodies</td>
<td>0.073±0.012</td>
<td>77±7</td>
</tr>
<tr>
<td>Choroid</td>
<td>0.045±0.003</td>
<td>44±2</td>
</tr>
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Values are the mean±SD from three separate experiments.
high \( M_r \) radiolabeled band varied from 20 to 30% of the 66,000 \( M_r \) protein determined by densitometric traces of the autoradiograph. However, the electrophoretic and autoradiographic result of ANP receptor in choroid yielded different pattern. A component of \( M_r \) 130,000 was intensely labeled with the crosslinking reagent, DSS. In addition to the intense 130,000 \( M_r \) protein band, a less intense 66,000 \( M_r \) radiolabeled band was also faintly seen in Fig. 3B. The relative proportion of this lower \( M_r \) radiolabeled band varied from 40–60% of the 130,000 \( M_r \) protein. Radioactivity was incorporated into the same two polypeptide bands to different extents in the ciliary bodies and choroid under the nonreducing condition. In the presence of reducing reagent, however, the 130,000 \( M_r \) radiolabeled proteins migrated in the gel to the 66,000 \( M_r \) position (Fig. 3, lanes 3 and 7). These molecules are similar to those reported for other target tissues (3, 7, 17, 18, 20).

DISCUSSION

The present studies demonstrated the presence of two classes of binding sites for ANP in the hog ciliary bodies and choroid and suggested the involvement of ANP in the regulation of aqueous humor flow and intraocular pressure.

The values obtained above show that our data are consistent with the assumption that 130,000 \( M_r \) protein represents the low affinity receptor site, and 66,000 \( M_r \) protein the high affinity receptor as suggested by the equilibrium binding studies. The presence of two binding sites with different affinity was suggested by several workers (8, 10, 14). However, the consistency of the molecular size and subunit structure with the different affinity observed by equilibrium binding studies has not been demonstrated.

The curvilinearity of Scatchard plot may result from absolute negative cooperativity
assuming that the 130,000 \( M_r \) protein is composed of two 66,000 \( M_r \) subunits and has two interacting binding sites for ANP. Similar discussions have been reported for other disulfide-linked cell surface proteins such as insulin receptor (19).

Interesting finding is that most of the ANP receptor in ciliary bodies exists in non disulfide-linked form in the native state, and that the predominant binding sites in choroid exist in a high \( M_r \) form. Although these low \( M_r \) subunits bind to ANP with high affinity, and high \( M_r \) subunits represent the low affinity binding sites, it is not clear which subunits function more actively in the regulation of water/sodium transport.

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REFERENCES


