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CHARACTERIZATION OF SPECIFIC RECEPTORS FOR ATRIAL NATRIURETIC FACTOR ON CULTURED BOVINE BRAIN CAPILLARY ENDOTHELIAL CELLS

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SUMMARY: A specific receptor for rat atrial natriuretic factor (rANF) was identified on primary cultures of bovine brain capillary endothelial cells (BBCEC's). Cultured BBCEC's have been developed in our laboratory as an *in vitro* model of the blood-brain barrier. The binding of [^{125}I]-rANF was rapid, reversible, saturable and unaffected by the presence of hormonal peptides such as insulin, vasopressin and angiotensin II. Scatchard analysis of competitive binding studies indicated the presence of a single class of binding sites for rANF with a dissociation constant of 400 pM and maximal binding capacity of 52 fmol/mg total cell protein. Binding of [^{125}I]-rANF was inhibited to varying degrees by atriopeptins I-III with atriopeptin III being the most potent and atriopeptin I being the least potent. BBCEC's also rapidly internalized [^{125}I]-rANF (100% after 60 min at 37°C) by a temperature-dependent process.

Since the discovery in 1981 (1) that atrial extracts produce potent diuretic and natriuretic effects, the mechanism of action and the identification of specific receptor binding sites for atrial natriuretic factor (ANF) and atriopeptins (AP's) in the kidney, adrenal gland, vasculature and brain have been widely explored (2,3,4,5). In its active form, ANF is a 28 amino acid peptide that acts rapidly (within 2-3 min) in response to increased cardiac output, blood pressure and sodium content (2). Of particular interest in our laboratory is the recent identification of ANF receptors in intact brain microvessels (6). This observation suggests the possibility that ANF is involved in regulating cerebrovascular pressure and permeability of water and ions through the blood-brain barrier (BBB).

Recently, our laboratory developed an *in vitro* model of the BBB, which consists of primary cultures of bovine brain capillary endothelial cells (BBCEC's) (7). This vascular endothelial cell culture system was characterized morphologically, biochemically and

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histochemically and was shown to possess the features of the BBB (7,8).

In the studies described below, we have identified a specific receptor for [^{125}I]-rANF on primary cultures of BBCEC's. This observation serves as the first step toward elucidating the physiological role of ANF in the regulation of cerebrovascular pressure and the permeability of water and ions through the BBB.

MATERIALS AND METHODS

Chemicals: Reagents and chemicals were obtained from the following sources: [^{125}I]-rANF (2000 Ci/mMol), Amersham (Arlington Hgts., IL); synthetic unlabelled rANF, Boehringer Mannheim Biochemicals (Indianapolis, IN).; AP I, AP II, AP III, insulin, angiotensin II and vasopressin, Sigma Chemical Company (St. Louis, MO). All other chemicals were reagent grade or better.

Isolation and Culture of BBCEC's: Capillary endothelial cells were isolated from the cerebral gray matter of bovine brains as described by Audus and Borchardt (7). After isolation, approximately $5x10^5$ cells were seeded on 35 mm Corning culture dishes. Prior to seeding, the dishes were coated with rat tail collagen and fibronectin, then UV sterilized for 90 min. After 10-12 days in culture, all dishes were inspected by microscopy to determine the degree of confluency. The morphological, biochemical and histochemical characteristics of the cultured BBCEC's have been reported earlier (8), and this *in vitro* model system was shown to possess all the features of the BBB including tight intercellular junctions, the lack of membrane fenestrations and the presence of γ-glutamyl transpeptidase, alkaline phosphatase and Factor VIII antigen.

Binding and Internalization of [^{125}I]-ANF by Cultured BBCEC's: Prior to conducting the protein binding and internalization studies, cell monolayers were washed two times with assay buffer (120 mM NaCl, 4.7 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM $MgSO_4$, 25 mM NaH_2CO_3, 0.05 mM NaEDTA, 1.2 mM NaH_2PO_4, 10 mM HEPES, pH 7.4) and then incubated at 37^o for 30 minutes. The time course of total [^{125}I]-rANF binding was measured by exposing the cell monolayers to assay buffer containing 0.1 nM [^{125}I]-rANF at 4^{o}C or 37^{o}C. After specified times, the cells were washed three times with fresh, ice cold assay buffer and removed from the dishes with two, 1 ml aliquots of 0.2 N NaOH. The radioactivity of the entire cell suspension was measured using a Beckman 5500 gamma counter followed by protein determination (Bio Rad protein assay). The non-specific binding of [^{125}I]-rANF was measured by adding 1 μM unlabelled rANF to separate sets of cells. Specific binding was calculated by subtracting the amount of non-specific binding from the total binding for each time point.

Competitive displacement experiments were conducted as described above with [^{125}I]-rANF (0.1 nM) except that varying concentrations (0-5 nM) of non-radioactive rANF or 1 μM concentrations of atriopeptins I-III, insulin, vasopressin or angiotensin II were added to the assay buffers. After 30 min incubation time at 4^{o}C, the cells were washed and treated as described above. The binding parameters (dissociation constant (Kd), maximum binding capacity (Bmax) and non-specific binding) for rANF displacement were obtained using the LIGAND fitting program (9). The maximal amounts of [^{125}I]-rANF that bound to BBCEC's

in the presence of the AP's and hormonal peptides were calculated as the percent of the total amount of [^{125}I]-rANF that was initially added to the cell monolayer.

Internalization of [^{125}I]-rANF was studied by removing the surface bound [^{125}I]-rANF using an acid wash technique (10). After initial binding for specified times at 37°C, the cells were washed two times with fresh, ice cold assay buffer, then exposed to 500 mM NaCl, 200 mM acetic acid, pH 3.0 for 3 min. The cells were subsequently washed three more times with assay buffer before the remaining radioactivity and total cell protein were measured (Bio Rad protein assay).

RESULTS

The time courses of [^{125}I]-rANF binding to monolayers of BBCEC's at 4°C and 37°C are shown in Figure 1. The binding of [^{125}I]-rANF to BBCEC's was rapid and reached a plateau after 20 min with 52 fmol/mg total cell protein (10%/mg total cell protein) bound at 4°C. At 37°C, the amount of binding peaked after 20 min and the maximun binding capacity decreased to approximately 20 fmol/mg total cell protein or 4%/mg total cell protein.

Figure 2 shows the binding of [^{125}I]-rANF at 4°C as a function of unlabelled rANF in a competitive displacement type of experiment. The binding was reversible and maximally displaced by the addition of 5 nM rANF. The Kd for ANF binding to the BBCEC receptor was 400 pM. Scatchard analysis of the binding data was linear, indicating a single

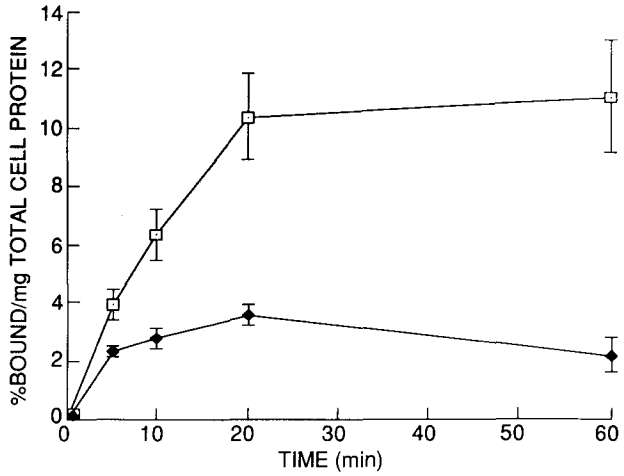

Figure 1. **Time Course of [^{125}I]-rANF Specific Binding to BBCEC Monolayers.** The time course of [^{125}I]-rANF binding was measured by exposing 0.1 nM [^{125}I]-rANF to BBCEC monolayers for the indicated times at 4°C (□) or 37°C (♦). After each time period, the cells were washed three times with fresh assay buffer and the fraction of rANF bound to the cells was measured. Non-specfic binding (measured in the presence of 1 μM excess ANF) was subtracted from the total binding and normalized by the total cell protein content. Each data point represents the mean ± SD of 3-5 experiments.

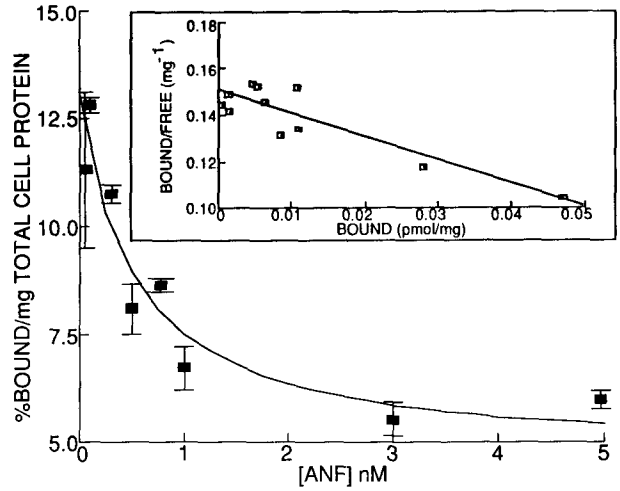

Figure 2. **Competitive Displacement and Binding of [^{125}I]-rANF to BBCEC Monolayers.** The fraction of [^{125}I]-rANF bound to BBCEC's was measured after 30 min at 4°C in the presence of the indicated rANF concentrations. The Kd and the Bmax were 400 pM and 52 fmol/mg total cell protein, respectively. The inset shows the Scatchard plot of the displacement data. Each data point represents the mean ± SD 5-7 experiments.

class of binding sites with a Bmax of 52 fmol/mg total cell protein existing on the BBCEC's (Figure 2 inset). Fitting of a two site model to the data was not significantly better than the single site model.

The fraction of the total [^{125}I]-rANF bound to BBCEC's that is internalized as a function of time at 4°C and 37°C is shown in Figure 3. The amount of acid resistant [^{125}I]-rANF increased from

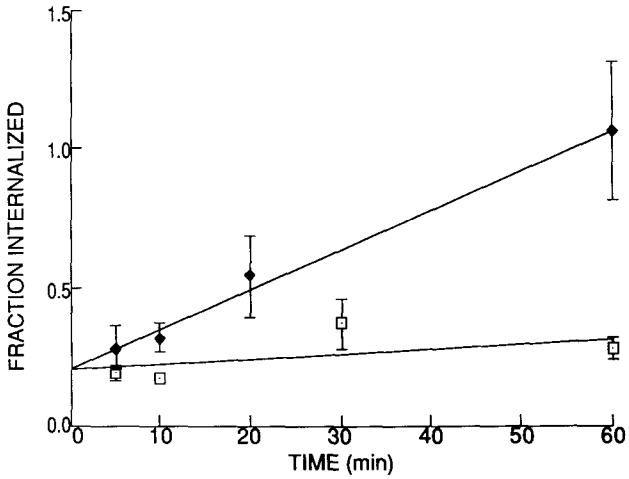

Figure 3. **Internalization of [^{125}I]-rANF by BBCEC Monolayers.** The amount of [^{125}I]-rANF that remained bound to BBCEC monolayers after acid exposure was measured at 4°C (□) or 37°C (♦). The data is presented as the fraction of the total [^{125}I]-rANF bound at each temperature that was internalized as a function of time. Each data point represents the mean ± SD of 3 experiments.

TABLE 1

EFFECT OF VARIOUS PEPTIDES ON [^{125}I]-rANF BINDING TO BBCEC's[a]

Peptide	Concentration (μM)	%B/mg[b]
-	-	15.1 ± 1.8[c]
Insulin	1.0	14.2 ± 3.4
Vasopressin	1.0	15.6 ± 0.6
Angiotensin II	1.0	16.1 ± 1.6
Atriopeptin I	1.0	13.7 ± 1.1
Atriopeptin II	1.0	11.8 ± 1.4[d]
Atriopeptin III	1.0	9.9 ± 1.9[d]
ANF	1.0	6.1 ± 1.7[d]

[a] Competitive binding experiments were conducted as described in the Materials and Methods section using [^{125}I]-rANF (0.1 nM).
[b] %B/mg - %[^{125}I]-rANF bound per mg total cell protein.
[c] Total percentage of [^{125}I]-rANF bound in the absence of competing peptides.
[d] Significantly different from control experiments with [^{125}I]-rANF alone ($P<0.05$).

approximately 25% after 5 min to nearly 100% after 60 min at 37°C. At 4°C, [^{125}I]-rANF internalization remained less than 30% of the amount internalized at 37°C for the entire 60 min time course.

In order to demonstrate the specificity of the ANF receptor on BBCEC's, competition experiments between [^{125}I]-rANF, related and unrelated peptides were conducted. The results (Table I) show that other hormonal peptides such as insulin, vasopressin and angiotensin II at concentrations up to 1 μM, do not significantly ($p< 0.05$) inhibit the binding of [^{125}I]-rANF (0.1 nM). However, rANF fragments, AP I, AP II, and AP III had varying degrees of affinity for the ANF receptor at 1 μM concentrations. All of the ANF fragments were less effective competitors of [^{125}I]-rANF when compared with the intact peptide.

DISCUSSION

Recently, the binding of ANF to bovine brain microvessel suspensions, which contain not only capillary endothelial cells but arterioles, venules and other associated cell material was demonstrated (6,11). The results presented here demonstrate that specific receptors for ANF are present on primary cell culture monolayers of BBCEC's which represent a pure population of the luminal capillary surface.

The binding of [^{125}I]-rANF was characterized by a rapid association at both 37°C and 4°C. At 4°C, the binding reached a plateau in 20 min

and remained at this level for at least 60 min. The binding at 37°C reached a peak after 20 min with slightly lower levels observed after 60 min. This suggests receptor down regulation or metabolism of [^{125}I]-rANF by the endothelial cells.

The Kd and the existence of a single class of binding sites for ANF observed in our studies, were comparable to the binding parameters measured for the intact microvessel (6) and other tissues such as vascular smooth muscle cells (12,13), adrenal cells and kidney tissue (14). The binding capacity of ANF for the cultured BBCEC monolayers (52 fmol/mg total cell protein) is nearly identical to the binding capacity of ANF to the intact microvessel (58 fmol/mg total cell protein) which suggests that the cultured monolayers are similar to the intact system with regard to the ANF receptor and serve as a good model to study the binding and effects of ANF on BBCEC's.

Internalization studies at 37°C indicated that a significant amount of ANF was taken up by the BBCEC's. Rapid and complete internalization of ANF is further evidence for cellular processing, regulation of ANF receptors on the cell surface and possible ANF transport from the blood to the brain.

The specific binding of ANF was demonstrated in experiments using other peptide hormones such as insulin, vasopressin and angiotensin II. These peptides were unable to displace [^{125}I]-rANF at concentrations as high as 1 μM. The binding was, however, effectively displaced by unlabelled rANF and to a lesser extent by the atriopeptins: AP I, AP II, and AP III. The rank order of atriopeptin binding displacement at equal concentration was consistent with the order of potency of these peptide fragments on rabbit aorta relaxation (15) and the displacement of ANF from smooth muscle cells (13). That is, AP III (5-28) was nearly as effective as ANF but further amino acid cleavage of ANF to AP II (5-26) and AP I(5-25) are much less effective in displacing [^{125}I]-rANF as well as producing a physiological effect. This suggests that the C-terminal portion of the molecule is important for binding to the receptor and in producing physiological effects.

Overall, these results are consistent with the existence of specific receptors on the luminal surface of BBCEC's for ANF. The presence of these receptors may have several roles, including: direct capillary effects, regulation of water and electrolyte permeability, communication between the circulation and the brain through binding to the receptor and/or the specific transport of ANF into the brain for neurologic or hormonal communication. Future experiments designed to elucidate the physiological role of the ANF receptor and the

possibility of BBCEC transport of ANF are currently ongoing in our laboratory.

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