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Identification of Endothelin Receptor Subtypes in Sheep Choroid Plexus

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Endothelin (ET) and its G-protein-coupled receptors are distributed in a wide variety of tissues, including the brain. In this study, we have identified and characterized the endothelin receptor subtypes in sheep choroid plexus. Competitive binding experiments using [^{125}I]ET-1 and the receptor subtype-selective ligands, ET-1, ET-3, BQ-123, Sarafotoxin 6c, and [Ala^{1,3,11,15}]ET-1 demonstrated the presence of both ET_A and ET_B receptor subtypes in the ratio of 30:70. In addition, a small fraction of the total binding sites exhibited affinities for ET-1 in the subpicomolar range. Chemical crosslinking of [^{125}I]ET-1 with *bis*(sulfosuccinimidyl)-suberate (BS^3) to choroid plexus membranes revealed the presence of two bands, with apparent molecular masses of 89 and 45 kDa, corresponding to the ET_A receptor, and three bands, with apparent molecular masses of 75, 58, and 33 kDa, corresponding to the ET_B receptor. Of considerable interest was the finding that dimers of the [^{125}I]ET-1-occupied ET_A receptor could be identified by crosslinking, as could apparent dimers and tetramers of [^{125}I]ET-1, but only when bound to receptor. In addition to mapping the distribution of ET receptors in sheep choroid plexus, our results strongly suggest that ET-1 binding to the ET_A receptor leads to dimer formation.

Key Words: Endothelin; receptor; choroid plexus.

Introduction

The endothelin (ET) peptide family is comprised of three members, ET-1, ET-2, and ET-3, which are encoded by three separate genes and differentially expressed in tissues of the periphery as well as in the central nervous system (CNS) *(1–4)*. The ET peptides, which share a marked degree of sequence identity with sarafotoxins, a family of peptide snake venom toxins, exhibit potent vasoactive, mitogenic, and neuroregulatory functions *(4)*.

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In mammalian cells, ETs exert their effects via two G-protein-coupled receptor subtypes, ET_A and ET_B *(6–8)*. The ET_A receptor subtype exhibits a higher affinity for ET-1 and ET-2 than ET-3 and sarafotoxin 6c (S6c), whereas the ET_B receptor subtype exhibits equal affinity for all three isopeptides and S6c. The receptors can also be distinguished by receptor subtype-selective ligands; BQ-123 is an ET_A-selective antagonist, whereas S6C and [Ala^{1,3,11,15}]ET-1 are ET_B-selective agonists. ET receptors are differentially expressed in a variety of tissues and cell types *(4)*. Both the ET_A and ET_B receptor subtypes activate a common intracellular signaling pathway, leading to a stimulation of phospholipase C and subsequent increase in the intracellular levels of inositol 1,4,5-trisphosphate (IP_3), diacylglycerol, and calcium *(4,5)*. Two additional receptors, ET_C and ET_{AX}, have been cloned and characterized *(9,10)*. ET_C binds ET-3 preferentially, whereas ET_{AX} binds ET-1 preferentially, but not BQ-123. Recently, an ET_B splice variant incapable of inducing intracellular IP_3 levels was cloned from human placenta *(11)*.

The ET peptides and their receptors can be detected in different regions of the brain *(2,3,12–17)*. ET-1 and ET-3 have been identified in cerebrospinal fluid at levels higher than in plasma *(18)*. ET_A and ET_B receptor subtypes have been identified in cultured brain endothelial cells, and glial cells primarily contain the ET_B receptor subtype *(19,20)*. Recent studies suggest that the ETs may exert neuromodulatory, neuroendocrine and trophic effects in the CNS. Several studies also suggest a role for ET in brain fluid balance *(21)*, and this may be related to the high density of specific [^{125}I]ET-1 binding sites present in the choroid plexus *(15,17)*. However, these binding sites have not been characterized, and the receptor subtype(s) in the choroid plexus have not been identified.

We have previously reported the presence of multiple binding sites in SCP cells, a sheep choroid plexus cell line *(22)*. In addition to the conventional ET_A receptor that mediates an increase in intracellular levels of IP_3, these cells also possessed binding sites with affinity in the subpicomolar range. In this study, we have characterized the ET receptors in sheep choroid plexus membranes by competitive binding assays using ET isoforms and subtype-

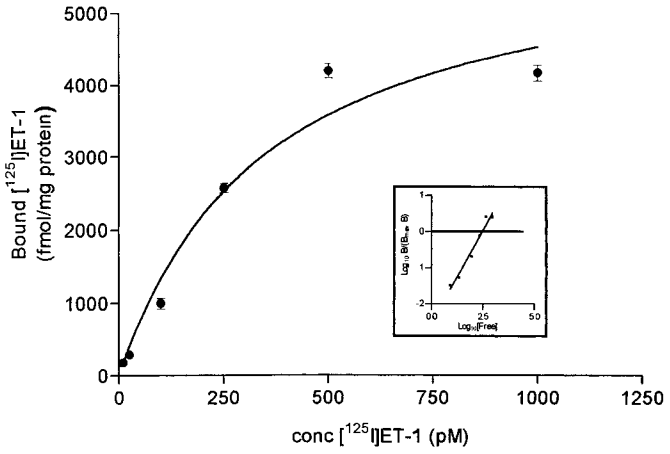

Fig. 1. Saturation (specific) binding of [^{125}]ET-1 to choroid plexus membranes. Membranes were incubated with increasing amounts of [^{125}I]ET-1 at 37°C for 1 h. Data are given as the mean ± SEM of two independent experiments. The inset shows the Hill plot of the same data. B, specific binding at each ligand concentration; B_{max}, maximum number of binding sites.

selective ligands, as well as by chemical crosslinking experiments. Our results show that a high density of both ET_A and ET_B receptors is present in the sheep choroid plexus. Binding sites with affinities for ET-1 and [Ala^{1,3,11,15}]ET-1 in the subpicomolar range are also present; however, these comprise a small fraction of the total receptors. Chemical crosslinking studies with *bis*(sulfosuccinimidyl)suberate (BS^3) of choroid plexus membranes suggest that the [^{125}I]ET-1-occupied ET_A receptors form dimers, and that the ET peptides themselves are capable of forming dimers and tetramers in the presence of receptor.

Results

[^{125}I]ET-1 binding to membrane preparations from sheep choroid plexus was optimized using between 10 and 50 µg of membrane protein and incubation times of 1 or 2 h at 37°C. There was no significant difference in specific binding of [^{125}I]ET-1 within this range of membrane protein or between the two incubation times (data not shown). Therefore, 10 µg of membrane protein and 1 h incubation at 37°C were chosen for all experiments.

Binding of [^{125}I]ET-1 to choroid plexus membranes is concentration-dependent and saturable, reaching a plateau at 500 p*M* (Fig. 1). Nonspecific binding, determined in the presence of 1 µ*M* ET-1, was between 2 and 9% of total binding. Nonlinear regression analysis of the binding data indicated the presence of an apparent single class of binding sites with a dissociation constant (K_d) of 0.3 n*M* and a receptor density of 5.8 pmol/mg membrane protein. The Hill coefficient was determined from the Hill plot of the data and was found to be 1.0 ± 0.1 (*see* inset to Fig.1).

To identify the relative proportion of ET receptor subtypes that are present in sheep choroid plexus, competitive

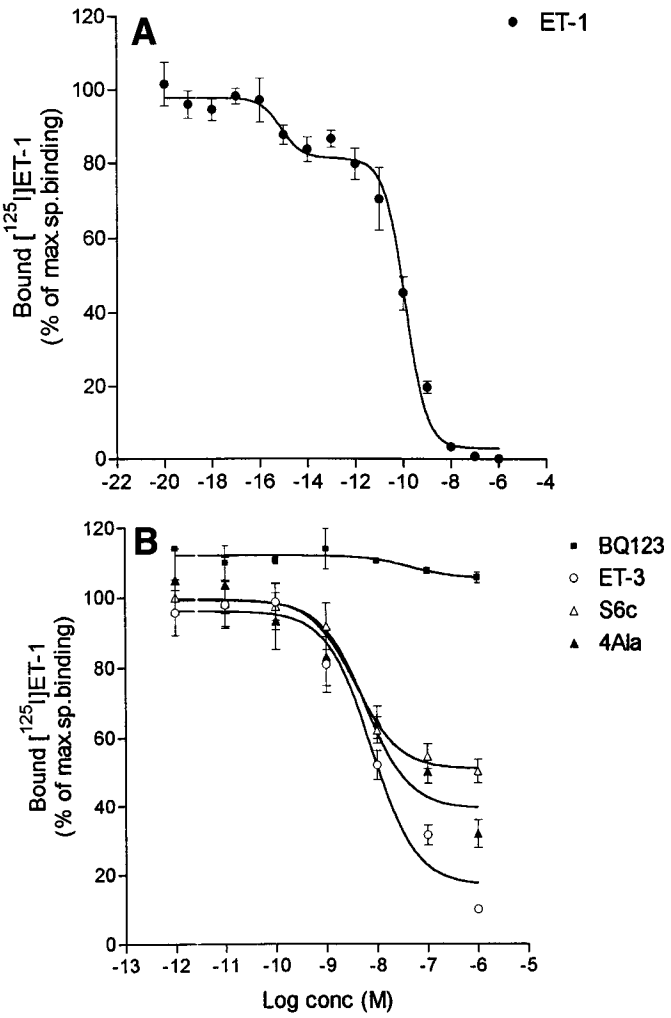

Fig. 2. Displacement of specific binding of [^{125}]ET-1 to choroid plexus membranes. **(A)** Competition with increasing concentrations of unlabeled ET-1. **(B)** Competition with increasing concentrations of unlabeled ET-3, BQ-123, [Ala^{1,3,11,15}]ET-1 (4Ala), and S6c. Specific binding obtained in the absence of competitor was normalized to 100%, and the results are presented as a percentage of this value. All assays were performed in duplicate, and the values are presented as mean ± SEM of 2 independent experiments for BQ-123 and four independent experiments for the other ligands.

binding experiments were performed with [^{125}I]ET-1 and unlabeled ET-1, ET-3, S6C, and [Ala^{1,3,11,15}]ET-1. Competition of 0.1 n*M* [^{125}I]ET-1 with increasing concentrations of unlabeled ET-1 indicated the presence of two binding components, the first characterized by an IC_{50} value of 0.8 f*M*, comprising approx 16% of the total binding sites, and the second exhibiting an IC_{50} of 0.1 n*M*, comprising about 79% of the total binding sites (Fig. 2A). Unlabeled ET-3 displaced [^{125}I]ET-1 with an IC_{50} of 7.9 n*M* (Fig. 2B). Both S6C and [Ala^{1,3,11,15}]ET-1 displaced [^{125}I]ET-1 with IC_{50} values of 3.8 and 6 n*M*, respectively (Fig. 2B). However, S6C was able to displace only 50% of the bound [^{125}I]ET-1 even at a concentration of 1 µ*M*. Similarly, [Ala^{1,3,11,15}]ET-1 displaced about 60% of the bound label, indicating that approx 50–60% of the receptors in the chor-

oid plexus belong to the ET_B receptor subtype. Therefore, the remaining 40–50% of the receptors are assigned to the ET_A receptor subtype, although BQ-123 did not displace the bound [^{125}I]ET-1 even at a concentration of 1 μ*M*.

In order to further characterize this component which did not bind BQ-123, competitive binding experiments with unlabeled ET-1 and ET-3 were performed in the presence of S6C or [Ala^{1,3,11,15}]ET-1 to block the ET_B receptors. The addition of 0.1 μ*M* S6C decreased [^{125}I]ET-1 binding to membranes by nearly 40% compared to the control value obtained in the absence of S6C (Fig. 3A). The ET-1 competition curve indicated the presence of two binding sites with IC_{50} values of 0.1 p*M* and 0.5 n*M*. The ET-3 competition curve showed only a single binding site with an IC_{50} value of 0.15 μ*M*. Similarly, in the presence of 0.1 μ*M* [Ala^{1,3,11,15}]ET-1, binding of [^{125}I]ET-1 is decreased by 55% (Fig. 3B). Competition with unlabeled ET-1 revealed the presence of two classes of binding sites with IC_{50} values of 0.4 p*M* and 0.6 n*M*. Unlabeled ET-3 competes with the bound [^{125}I]ET-1 to reveal a single binding site with an IC_{50} of 0.1 μ*M*. Surprisingly, in the presence of either S6C and [Ala^{1,3,11,15}]ET-1, BQ-123 is able to compete the bound [^{125}I]ET-1 with IC_{50} values of 6.4 and 14 n*M*, respectively. These binding data were characteristic of the ET_A subtype, even though BQ-123 was unable to bind this component under the standard conditions of the competitive binding assay. This result was interesting and suggested that BQ-123 could only bind the ET_A receptor subtype when the ET_B receptor subtype was blocked (compare Fig. 2B with Fig. 3C).

To characterize further the ET_B receptor subtype, competitive binding experiments were performed in the presence of 1 μ*M* BQ-123 (Fig. 4). Competition with unlabeled ET-1 elucidated two binding sites with IC_{50} values of 2.4 p*M* and 0.8 n*M*. Competition with [Ala^{1,3,11,15}]ET-1 also defined two binding sites, with IC_{50} values of 6.7 p*M* and 1.3 n*M*, whereas both ET-3 and S6C showed only a single binding site with IC_{50} values of 1.2 and 2.5 n*M*, respectively. These results suggest that in addition to the normal ET_B receptor subtype, a second binding site of higher affinity for ET-1 and [Ala^{1,3,11,15}]ET-1 exists in the choroid plexus.

Since the saturation binding data suggested a K_d of 0.3 n*M* for the ET receptors, and the competitive binding experiements demonstrated that the ET_A and ET_B receptors have similar IC_{50} values, about half of each receptor subtype would be expected to be occupied at the concentration of 0.1 n*M* [^{125}I]ET-1, which was used in the experiments. We postulated that BQ-123 could, in fact, bind ET_A receptors, but that the displaced [^{125}I]ET-1 could then bind to the unoccupied ET_B receptors; the net result would be that the bound CPM remains the same. However, if a saturating concentration of [^{125}I]ET-1 is used so that all of the receptors are occupied, then the bound [^{125}I]ET-1 that is displaced by BQ-123 would not be able to bind to ET_B receptor

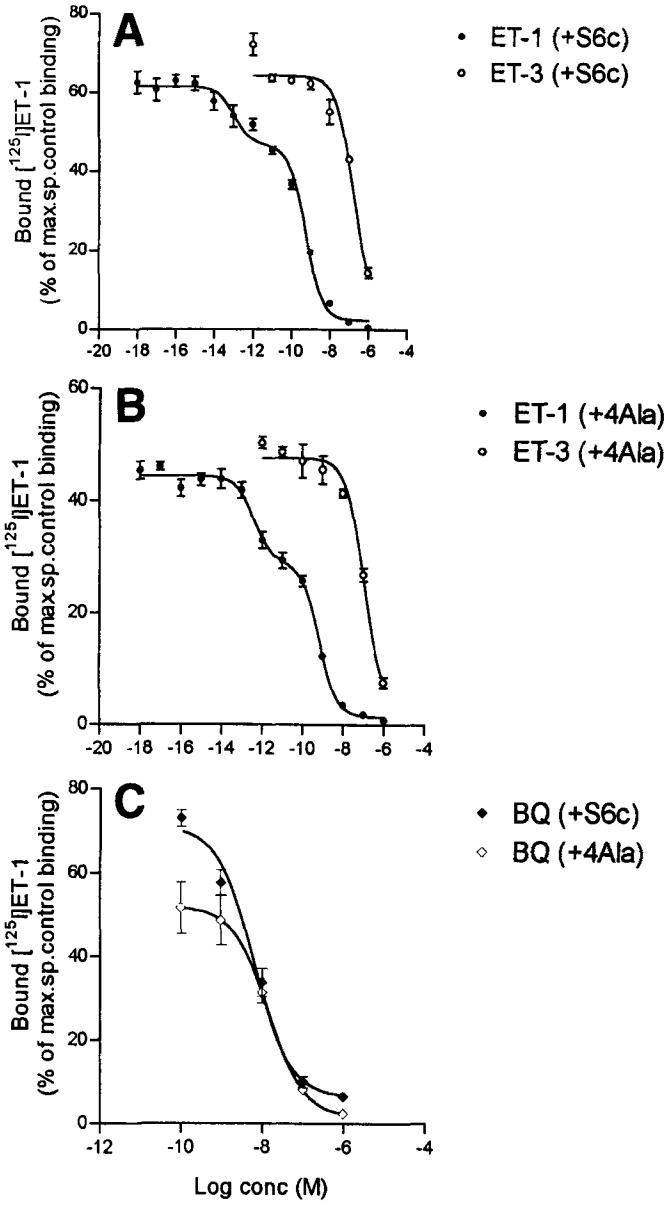

Fig. 3. Competition of specific binding of [^{125}I]ET-1 to choroid plexus membranes by unlabeled ET-1 and ET-3 in the presence of 0.1 μ*M* S6c and of [Ala^{1,3,11,15}]ET-1(4Ala) are shown in (**A**) and (**B**), respectively. (**C**) Competition of specific binding of [^{125}I]ET-1 to choroid plexus membranes by unlabeled BQ-123 in the presence of 0.1 μ*M* S6c and 4Ala. Specific binding in the absence of both S6c or 4Ala and unlabeled competitor was normalized to 100%, and the results are presented as a percentage of this value. All assays are performed in duplicate, and values are presented as mean ± SEM of 4 independent experiments for ET-1, 2 for ET-3, and 2 for BQ-123.

subtype and a decrease in the bound ET-1 should be observed. To test this hypothesis, we compared the specific binding obtained with 0.1 and 1 n*M* of [^{125}I]ET-1 (Table 1). At a ligand concentration of 0.1 n*M*, BQ-123 inhibited specific binding minimally (< 10%), whereas [Ala^{1,3,11,15}]ET-1 inhibited binding by over 40%. BQ-123 and [Ala^{1,3,11,15}]ET-1 together completely inhibited binding. At a saturating ligand concentration of 1 n*M*, as predicted,

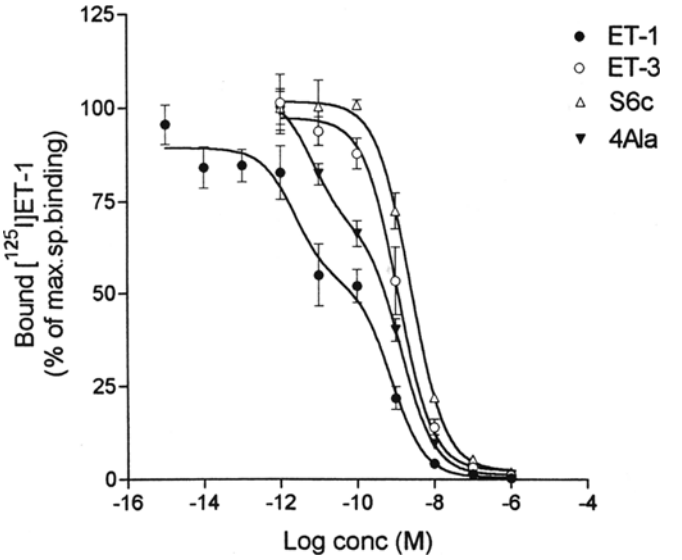

Fig. 4. Displacement of specific binding of [^{125}I]ET-1 to choroid plexus membranes by ET-1, ET-3, S6c, and [Ala^{1,3,11,15}]ET-1 (4Ala) in the presence of BQ-123. Specific binding in the absence of both BQ-123 and unlabeled competitor was normalized to 100%, and the results are presented as a percentage of this value. The values are presented as mean ± SEM of 4 experiments for ET-1, 3 for S6c, and 2 for ET-3 and 4Ala.

Table 1
Receptor Selective Inhibition
in the Presence of Subsaturating (0.1 n*M*)
and Saturating (1 n*M*) Levels of [^{125}I]ET-1[a]

Selective ligand	0.1 n*M*	1 n*M*
BQ-123	7	30
[Ala^{1,3,11,15}]ET-1	44	70
BQ-123 and [Ala^{1,3,11,15}]ET-1	93	97

[a]Choroid plexus membranes were incubated with either 0.1 or 1 n*M* [^{125}I]ET-1 in the presence of 1 μ*M* BQ-123 or [Ala^{1,3,11,15}]ET-1 at 37°C for 1 h. Inhibition is expressed as the percent of specific CPM displaced in the presence of the ligand relative to the total specific CPM bound in the absence of ligand.

specific binding is inhibited by 30% in the presence of BQ-123 and by 70% in the presence of 1 μ*M* [Ala^{1,3,11,15}]ET-1. The difference in the inhibition of specific binding observed with ligand concentrations of 0.1 and 1 n*M* [^{125}I]ET-1 in the presence of 1 μ*M* [Ala^{1,3,11,15}]ET-1 indicates that the [^{125}I]ET-1 displaced from the ET_B receptors can bind to the unoccupied ET_A receptors at subsaturating concentrations of ligand. This result suggests that both conventional ET_A and ET_B receptor subtypes are present in the choroid plexus in a ratio of 30:70 and that BQ-123 can bind effectively to the ET_A receptor subtype.

In order to examine if there were any possible molecular differences between the binding sites with nanomolar affinity and those with picomolar affinity, [^{125}I]ET-1 bound to sheep choroid plexus membranes was crosslinked and the products were separated by SDS-PAGE (Fig. 5A). In

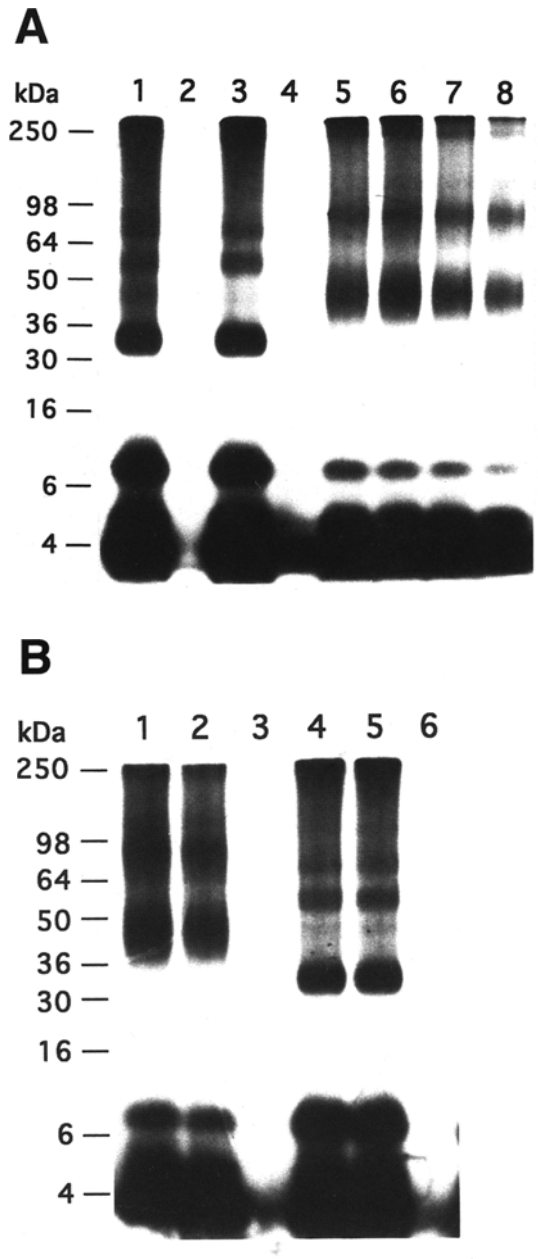

Fig. 5. Chemical crosslinking of [^{125}I]ET-1 to choroid plexus membranes. Crosslinked products were resolved by 10% SDS-PAGE and autoradiographed. The positions of prestained mol-wt markers are indicated. **(A)** Autoradiograph of crosslinked products obtained in the presence of various unlabeled competitors. Lane 1: control; lane 2: 1 μ*M* ET-1; lane 3: 1 μ*M* BQ-123; lane 4: 2 μ*M* ET-3; lane 5: 0.1 μ*M* S6c; lane 6: 1 μ*M* S6c; lane 7: 0.1 μ*M* [Ala^{1,3,11,15}]ET-1 (4Ala); lane 8: 1 μ*M* 4Ala. **(B)** Autoradiograph of crosslinked products generated in the presence 1 μ*M* 4Ala or BQ-123. Crosslinking was performed by incubating choroid plexus membranes with lane 1: 1 μ*M* 4Ala; lane 2: 1 μ*M* 4Ala and 0.1 n*M* ET-1; lane 3: 1 μ*M* 4Ala, and 1 μ*M* ET-1; lane 4: 1 μ*M* BQ-123 and 0.1 n*M* 4Ala; lane 5: 1 μ*M* BQ-123 and 0.1 n*M* ET-1; lane 6: 1 μ*M* BQ-123 and 1 μ*M* ET-1. Free ET-1 monomer is present at the bottom of the gel.

the presence of [^{125}I]ET-1, four predominant radiolabeled proteins with apparent molecular masses of 75, 58, 45, and

33 kDa were detected (lane1). The specificity of the crosslinking is indicated by the elimination of [^{125}I]ET-1 labeling by inclusion of unlabeled ET-1 (lane 2) or ET-3 (lane 4). In the presence of 1 μ*M* BQ-123, sufficient to block all ETA receptor subtypes, three cross-linked proteins of 75, 58, and 33 kDa were detected (lane 3). When the ETB receptor subtype is blocked with either S6C (lanes 5 and 6) or [Ala^{1,3,11,15}]ET-1 (lanes 7 and 8), two major crosslinked products of 89 kDa and 45 kDa were detected. The intensity of these bands in lane 1 is lower, presumably because the ET_A receptor subtype constitutes only 30% of the total receptors. In the presence of both BQ-123 and S6C or of BQ-123 and [Ala^{1,3,11,15}]ET-1, all of the crosslinked products are eliminated, indicating that they are specific to the receptor.

To determine if the picomolar affinity binding site identified by the competitive binding experiments could be attributed to one of the cross-linked bands identified above, additional cross-linking experiments were performed with a lower concentration of unlabeled ET-1 (0.1 n*M*) in the presence of 1 μ*M* [Ala^{1,3,11,15}]ET-1 to block ET_B receptors (Fig. 5B, lane 2). However, there was no change when compared to crosslinked products obtained with 1 μ*M* [Ala^{1,3,11,15}]ET-1 alone (lane 1). Crosslinking experiments were also performed to determine if a protein band could be attributed to the picomolar affinity binding site that was identified as being specific for ET-1 and [Ala^{1,3,11,15}]ET-1, but not S6C or ET-3. Competition with [Ala^{1,3,11,15}]ET-1 or 0.1 n*M* ET-1 (lanes 4 and 5), in the presence of BQ-123 to block the ET_A receptor subtype, did not eliminate any of the crosslinked products observed in the absence of unlabeled ET-1 or [Ala^{1,3,11,15}]ET-1 (Fig. 5A, lane 3). In addition to the high-mol-wt crosslinked products, discrete low-mol-wt products that migrated slower than free [^{125}I]ET-1 were detected. The estimated sizes of these products are 5 and 10 kDa, and these products were not formed when crosslinking was performed in the absence of membrane (data not shown). Thus, they appear to represent crosslinked [^{125}I]ET-1 molecules that were bound to receptor.

Discussion

The binding and crosslinking studies presented herein demonstrate the presence of both ET_A and ET_B receptor subtypes in the sheep choroid plexus. In addition, Northern blot analysis of poly(A)$^+$ RNA prepared from choroid plexus tissue confirmed the presence of both ET_A and ET_B receptors (data not shown). The ET_B receptor subtype comprises 70% of the total receptors, consistent with previous *in situ* hybridization studies that had shown the predominance of ET_B mRNA in brain *(19)*. In addition, competitive binding studies revealed the presence of two additional sites with binding affinities for ET-1 in the picomolar range. One of these sites binds [Ala^{1,3,11,15}]ET-1, but not S6c. These sites, however, constitute a small fraction of the total binding sites.

Our previous studies with SCP cells, a cell line derived from sheep choroid plexus, showed the presence of the conventional ETA receptor with an IC_{50} of 0.6 n*M* and two additional binding sites with affinities of 0.1 p*M* and 5 a*M* for ET-1 *(22)*. Choroid plexus tissue contains the conventional ET_A receptor and the binding site with an affinity of 0.1 p*M*. However, the third binding site, with an IC_{50} value of 5 a*M* for ET-1, was not identified. One possible explanation for this difference is that the fraction of this site in the choroid plexus, which contains multiple cell types and both ET_A and ET_B receptors, is too small compared to SCP cells, which comprise a single cell type and only the ET_A receptor subtype.

Surprisingly, the very high-affinity binding site (IC_{50} = 0.8 f*M*) detected in competition studies with ET-1 was not detected when competitive binding with ET-1 was performed after ET_B receptors were selectively blocked. This suggests that S6c and [Ala^{1,3,11,15}]ET-1 can block this high-affinity site or that ET_A and ETB receptors interact cooperatively to generate a high-affinity binding site. The latter seems unlikely, since the Hill coefficient determined from the saturation binding studies does not indicate any cooperativity; however, low concentrations in the femitomolar range cannot be directly assessed using saturation binding.

Chemical crosslinking with [^{125}I]ET-1 and choroid plexus membranes confirmed the presence of both ET_A and ET_B receptors. Crosslinking studies performed with membranes from rat and porcine cerebellum and human cerebral cortex has demonstrated that the size of the ET_B receptor subtype ranges from 47–53 kDa *(23–25)*. In addition, a smaller product of 31–38 kDa was also detected. The purified ET_B receptor from bovine lung contains two forms with apparent M_rs of 52 and 34 kDa; the latter was generated by a specific metalloprotease-catalyzed cleavage of the intact receptor in the amino-terminal extracellular domain between Ala-79 and Gly-80 of the bovine ET_B receptors *(26,27)*. Therefore, the 58-kDa crosslinked product detected with choroid plexus membranes most likely represents the ET_B receptor, and the 33-kDa product a proteolytic product. The nature of the 75-kDa product is not clear, although it may represent a heterodimer containing the full-length receptor and the proteolytic product. Since the competition binding experiments revealed the presence of a binding site that bound ET-1 and [Ala^{1,3,11,15}]ET-1 with IC_{50} values of 2.4 and 6.7 p*M*, respectively, it was of interest to determine if any of the crosslinked products corresponded to this site. However, competition with unlabeled ET-1, at a concentration that would only displace bound ET-1 from the p*M* affinity binding site, did not eliminate any of the crosslinked products.

Only two crosslinked products were detected that correspond to the ET_A receptor subtype. The size of the 45-kDa product is consistent with the size of the receptor reported in COS-7 cells transfected with the ET_A receptor cDNA

(*28*; unpublished results). Crosslinking studies with the ET_A receptor in A-10 cells, C6 glial cells, chick cardiac membranes, and rat lung membranes have generally yielded a single band with molecular sizes ranging from 45–73 kDa *(29–31)*. Interestingly, the size of the larger product we detected (89 kDa) is consistent with a dimer. Several structural studies, including cross-linking, have suggested that G-protein-coupled receptors can form dimers *(32–34)* and a recent study clearly demonstrated that β_2-adrenergic receptors formed dimers *(35)*. The functional significance of the dimer formed with G-protein-coupled receptor is not clearly understood. One possibility is that the picomolar affinity binding site, with the characteristics of the ET_A receptor, is a result of dimerization of the normal high-affinity receptor. However, competition with 0.1 n*M* unlabeled ET-1, which would only displace [^{125}I]ET-1 from this site, had no effect on the 89 kDa crosslinked product.

Another intriguing observation was the presence of discrete low-mol-wt crosslinked products. These products migrate slower than the free [^{125}I]ET-1, and their estimated sizes are 5 and 10 kDa. These size estimates should be treated with caution because of the inherent inaccuracy of the gel system in this range. Nonetheless, these sizes are consistent with that of an ET-1 dimer and tetramer. These products are formed only in the presence of added membrane, indicating that they are receptor-dependent. Perhaps endothelin peptides bind to receptors as dimers, or single peptide–receptor complexes can form dimers resulting in the detection of both peptide and receptor dimers. Clearly, further studies are warranted to confirm the presence of both receptor and peptide dimers.

In situ hybridization studies had previously shown that ET_B mRNA is present in the epithelial cells of the choroid plexus *(19)*. Our study has confirmed the presence of the ET_B receptor protein in this tissue, and, in addition, has shown that the choroid plexus also contains the ET_A receptor subtype. Since the choroid plexus is a highly vascularized tissue, it is possible that the ET_A receptors are localized to the blood vessels. Although the role of ET receptors in the choroid plexus is not understood, in vivo studies suggest that they may regulate sodium and water reabsorption *(21)*.

In summary, we have shown, using receptor-selective ligands and chemical crosslinking, that sheep choroid plexus contains both the ET_A and ET_B receptor subtypes, with a greater than twofold excess of the latter. Interestingly, [^{125}I]ET-1, as revealed by chemical crosslinking, can bind to higher-order structures of both receptor subtypes or, consistent with many ligand–receptor systems, promote association of peptide–receptor complexes.

Materials and Methods

Materials

Sheep choroid plexus tissue was purchased from Pel-Freez Biologicals (Rogers, AR) and shipped frozen to our laboratory. [^{125}I]ET-1 (2200 Ci/mmol) was purchased from Dupont NEN (Boston, MA). ET-3 was a product of Bachem (Torrance, CA); ET-1, S6c, BQ-123, and [$Ala^{1,3,11,15}$]ET-1 were obtained from Peptides International (Louisville, KY) and Peninsula Labs (Belmont, CA). BS^3 was from Pierce (Rockford, IL). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Membrane Preparation

Choroid plexus membranes were homogenized in 10 vol of cold homogenization buffer containing 50 m*M* Tris-HCl, pH 7.5, 0.25 *M* sucrose, 2 μg/mL aprotinin, 1 μ*M* pepstatin, 0.1 m*M* phenylmethylsulfonylfluoride, and 3 m*M* EDTA using a polytron. The homogenate was centrifuged at 1000*g* for 20 min, and the resulting supernatant was centrifuged at 50,000*g* for 20 min at 4°C. The pellet was resuspended in homogenization buffer and centrifuged again at 50,000*g* for 20 min. The final pellet was resuspended in 50 m*M* Tris-HCl, pH 7.5, containing protease inhibitors, and the protein concentration was determined using the BCA assay (Pierce). The membranes were stored in aliquots at –70°C at a concentration of approximately 10 mg protein/mL

Receptor Binding Assays

Binding of [^{125}I]ET-1 was performed in duplicate with 10 μg of membranes in 0.5 mL binding buffer containing 50 m*M* Tris-HCl, pH 7.5, 0.3% bovine serum albumin (BSA), and 5 m*M* EDTA. After incubation at 37°C for 1 h, the membranes were pelleted by centrifugation at 14,000*g* for 10 min at 4°C and washed twice in binding buffer. The membrane pellet was solubilized in 0.1 *N* NaOH and the bound radioactivity was measured using a γ-counter.

Saturation binding experiments were performed using increasing concentrations (10–1000 p*M*) of [^{125}I]ET-1; nonspecific binding was determined in the presence of 1 μ*M* of unlabeled ET-1. Competitive binding experiments were carried out with 0.1 n*M* [^{125}I]ET-1 in the absence (total binding) and presence of increasing concentrations of various unlabeled ligands. Competitive binding experiments were also performed with 0.1 n*M* [^{125}I]ET-1 either in the presence of 1 μ*M* BQ-123 and increasing concentrations of unlabeled ET-1, ET-3, S6c and [$Ala^{1,3,11,15}$]ET-1, or in the presence of 0.1 μ*M* S6c or [$Ala^{1,3,11,15}$]ET-1 and increasing concentrations of unlabeled ET-1, ET-3, and BQ-123.

Data Analysis

The data presented are an average of two to four experiments, and analysis was performed by the Prism software (GraphPad Software, San Diego, CA). Saturation binding experiments were analyzed by nonlinear regression analysis of untransformed data. Competition curves were also analyzed by nonlinear regression using one-site and two-site models, and the best fit was chosen with a $P < 0.05$ and $R^2 > 0.99$. In addition, the two-site model was considered to be significant only when the 95% confidence intervals of the IC_{50} values did not overlap.

Crosslinking Analysis

Choroid plexus membranes (50 µg) were incubated with 0.1 n*M* [^{125}I]ET-1 in 0.3 mL buffer containing 50 m*M* HEPES, pH 7.5, and 0.1% BSA in the absence or presence of various unlabeled peptides (ET-1, ET-3, BQ-123, S6c, and [Ala^{1,3,11,15}]ET-1). After incubation at 37°C for 1 h, the reaction mixture was centrifuged at 10,000*g* for 10 min at 4°C. The pellet was washed five times with 50 m*M* HEPES, pH 7.5, resuspended in the same buffer, and the crosslinking agent, BS^3, was added to a final concentraion of 1 m*M*. Following incubation at room temperature for 1 h, the reaction was quenched by the addition of Tris-HCl, pH 7.5, to a final concentration of 40 m*M*, centrifuged at 10,000*g* for 10 min, and the resulting pellet was resuspended in 20 µL of sample buffer (50 m*M* Tris-HCl, pH 6.8, 12% glycerol, and 4% SDS). The samples were resolved by electrophoresis on a 10% SDS-polyacrylamide gel under nonreducing conditions using the Tris-tricine buffer system (*36*). The gel was fixed for 20 min in 40% methanol/10% acetic acid, dried, and autoradiographed with an intensifying screen at –70°C.

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