Acta Med. Nagasaki 38:86 – 90

Receptor Autoratiographic Evidence for Specific ^{125}I-Endothelin-1 Binding Sites in the Rat Eye

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Specific ^{125}I-endothelin-1 (^{125}I-ET-1) binding sites were investigated in the rat eye, using receptor autoradiographic techniques. ^{125}I-ET-1 was specifically bound to the rat eye sections, with a slow association rate, as evidenced by kinetic experiments. The radioligand binding reached a maximum at 48hr of incubation and a plateau was maintained for up to 72hr. No degradation of ^{125}I-ET-1 during incubation was observed at 72hr. Specific ^{125}I-ET-1 binding sites were localized in areas corresponding anatomically to the cornea, the iris, the retina, the choroid and the sclera. ^{125}I-ET-1 binding to these sections was monophasically inhibited by unlabeled ET-1 with dissociation constant (K_d) of 128pM, whereas unlabeled ET-3, a member of the ET family peptides, biphasically inhibited binding with low affinity, inhibition constant (K_i) of 7.06nM, and high affinity, K_i of 53pM. This evidence for specific ^{125}I-ET-1 binding sites supports the physiological significance of the ET family peptides in the rat eye.

Introduction

Endothelin-1 (ET-1) and ET-3, members of the ET family peptides[1,2], are functional peptides in the eye regulating intraocular pressure, ocular blood vessel tone, and iris smooth muscle tone[3,4]. ET-1 gene expression in the rat iris was noted using in situ hybridization techniques with a radiolabeled ET-1 complementary RNA probe[5]. ET-1-and ET-3-like immunoreactivities were also histochemically evident in rabbit ocular areas corresponding anatomically to the iris and to the ciliary body[3]. The peptides presumably exert physiological functions in the eye, by interacting with specific receptors, classified recently as two subtypes; the ET_A receptor[6], preferentially recognized by ET-1, and the ET_B receptor[7], a non-selective subtype.

Although specific binding sites for ^{125}I-ET-1, candidates of physiologically active receptors, have been detected within the eye[5,8], much less is known of characteristics of the ET receptor in this organ. Using the quantitative receptor autoradiography[9,10] with radioluminography[11,12], we investigated specific ^{125}I-ET-1 binding sites, candidates of physiologically active receptors for the ET family peptides, in the rat eye.

Materials and Methods

Materials

^{125}I-ET-1 was purchased from the New England Nuclear, U. S. A., and peptides used were from the Peninsula Lab., U. S. A. and the Peptide Institute, Japan. Drugs were purchased from the Sigma Chemical Co., U. S. A. Male Wistar Kyoto rats weighing 250g were given standard chow (F-2, Funabashi Farm Co., Japan) and water ad libitum and were housed at 24℃,with lights on from 07:00 hr to 19:00 hr at the Laboratory Animal Center for the Biomedical Research, Nagasaki University School of Medicine, Japan. The rats were decapitated between 10:00 hr and 12:00 hr and eyes were rapidly removed and immediately placed in isopentane at -30℃. Frozen, 10-μm-thick eye sections were cut in a cryostat at -20℃, thaw-mounted onto gelatin-coated slides, and stored overnight under vacuum at 4℃.

Quantitative receptor autoradiography

Tissue sections were labeled in vitro with ^{125}I-ET-1 (specific activity, -81.4TBq/mmol) in 2.0ml of incubation box Briefly, after preincubation at room temperature (23℃) for 10min in the incubation buffer, consecutive tissue sections were incubated at 4℃ for 48hr with 32.6pM ^{125}I-ET-1, in the absence (total binding) or presence of increasing concentrations of unlabeled ET-1 and ET-3, ranging from 1.0pM to 1.0μM and 1.0pM to 10μM, respectively, in 50mM Tris-HCl buffer (pH 7.4) containing 100mM NaCl, 10mM EDTA -2Na, 1mg/ml bacitracin, 4μg/ml leupeptin, 2μg/ml chymostatin, 10μM phosphoramidon[13] and 0.2% (w/v) bovine serum albumin (proteinase-free). Following these incubations, the slides were washed 3 times (1min each) at 4℃ in 50mM Tris-HCl buffer (pH7.4), rinsed quickly in ice-cold distilled water, and then dried under a stream of cold air.

To quantitate ^{125}I-ET-1 bound to the sections, we used the computerized radioluminographic system with imaging plates coated with fine photostimulable phosphor crystals ($BaFBr:Eu^{2+}$)[11]. These dried, labeled sections were exposed to radioluminographic imaging plates (Type BAS-III) with

calibrated 10-μm-thick ^{125}I-standards ([^{125}I] micro-scales, Amersham, U. K.). The autoradiograms obtained were analyzed using the radioluminergic imaging plates system (Bio-imaging Analyzer BAS 2000, Fuji Photo Film Co., Japan). The values of photostimulated luminescence (PSL) directly obtained from the imaging plates by the computerized scanning system were converted to the bound radioactivity of the section, based on a comparison with standards curves for sets of standards rum on each imaging plate[12]. After quantitation, the sections labeled with the radioligand were also exposed to Hyperfilm-^{3}H (Amersham) to acquire autoradiograms. The films were developed at 4℃ for 7min, with a Kodak D19 developer (Eastman Kodak, U. S. A.).

In the initial experiment, we investigated the appropriate incubation time, under the conditions described above. Incubations were carried out at 4℃ for 30min, 1hr, 2hr, 6hr, 12hr, 24hr, 36hr, 48hr and 72hr, then the degradation of ^{125}I-ET-1 in the incubation buffer was checked using high-performance liquid chromatography (HPLC). One hundred μl sample of the incubation buffer with the radioligand was injected onto a HPLC system composed of a 6000A pump and reverse-phase μBondasphere C_{18} column (5μm C_{18}, 3.9 x 150mm, Waters, U. S. A.). ^{125}I-ET-1 was separated using a linear gradient of 20% to 40% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid, at a rate of 1.0ml/min. Fractions of 2.0ml were collected and the radioactivity was detected by γ-counting.

Data analysis

The data obtained by quantitative receptor autoradiographic studies were analyzed using the LIGAND computer program[14, 15]. Results were expressed as means ± S. E. Differences in the data were assessed by one-way analysis of variance (ANOVA) using the F test.

Results

In the initial experiments done at 4℃ with 26.7pM ^{125}I-ET-1, we found that specific ^{125}I-ET-1 binding to the rat eye sections reached a maximum at 48 hr, and a plateau was maintained for up to 72hr (Fig. 1). The radioligand was stable during the 72hr incubation time, as the HPLC analysis revealed no degradation of ^{125}I-ET-1 in the incubation buffer (Fig. 2). Hence, the following binding experiments were carried out at 4 ℃ for 48hr.

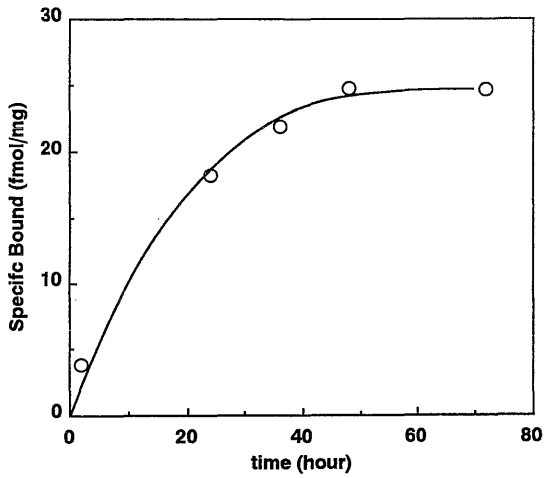

Fig. 1. Effect of incubation time on specific binding of ^{125}I-endothelin-1 (^{125}I-ET-1) to rat eye sections. Each point represents the mean of four determinations. Related tissue sections were incubated at 4 ℃ with 26.7pM ^{125}I-ET-1 in the absence or presence of 1.0μM unlabeled ET-1.

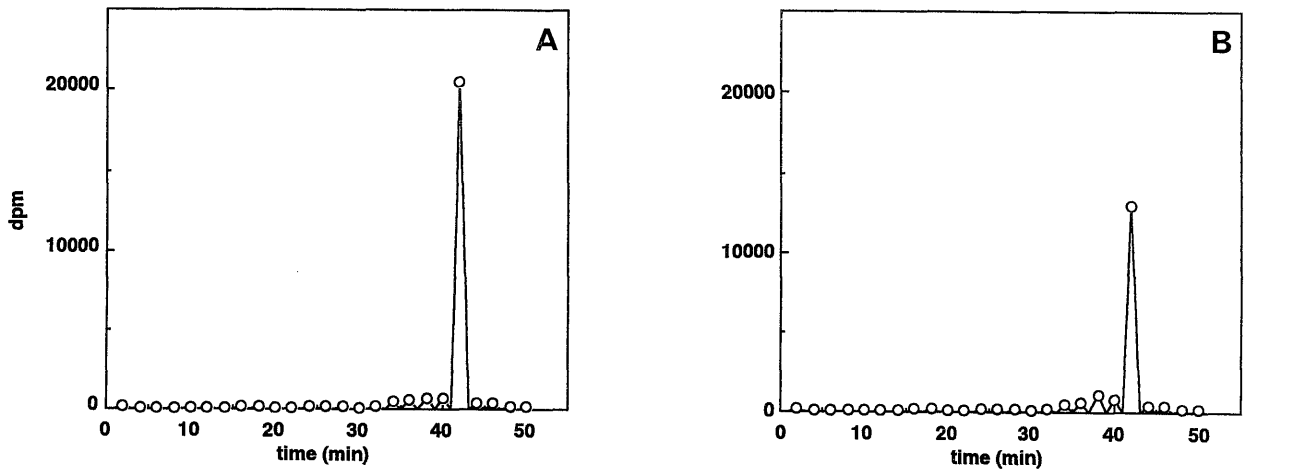

Fig. 2. High-performance liquid chromatographic analysis of ^{125}I-endothelin-1 (^{125}I-ET-1) before (A) and after (B) incubation of 72hr at 4 ℃. The sample was loaded on a reverse-phase μBondasphere C_{18} column (3.9 x 150mm) and eluted at 1.0ml/min with a linear gradient of 20% to 40% acetonitrile in 0.1% (v / v) trifluoroacetic acid. Fractions of 2.0ml were collected and the radioactivity was detected by γ-counting.

Autoradiographic localization of ^{125}I-ET-1 binding sites in the rat eye is shown in Fig. 3. ^{125}I-ET-1 richly labeled the cornea, the iris, the retina and the area anatomically corresponding to the choroid and the sclera (Fig. 3A). This localization is in accord with the findings of Koseki *et al.* (1989)[8] and MacCumber *et al.* (1989)[5]. Apparent specific binding concentrationes were calculated by subtracting non-specific binding concentrationes obtained at incubation with 32.6pM ^{125}I-ET-1 in the precence of 1.0μM ET-1 from total binding concentrations (Table 1). The highest densities were observed in the iris and the area of the choroid and the sclera Binding sites in the retina were divided into two areas, inner and outer parts, with high and moderate densities, respectively. A moderate density of binding was noted in the cornea. Unlabeled ET-1 at a concentration of 1.0μM completely inhibited ^{125}I-ET-1 binding to various areas, with the exception of the lens. ^{125}I-ET-1 slightly bound to the lens, however, the binding was not inhibited by 1.0μM ET-1 (Fig. 3B). We also calculated specific binding concentrations of ^{125}I-ET-1 remaining in the presence of 10μM ET-3. As shown in Table 1, interestingly, ET-3 most potently inhibited binding to the cornea. In this area, binding concentrations ramained at the presence of an excess amount, 10μM, of ET-3 was only 5.0% of specific binding concentration obtained in case of incubation with 32.6pM ^{125}I-ET-1. The peptide significantly inhibited binding to the iris and to the inner part of the retina, but 6.8% and 14.5% of specific binding remained, respectively. ET-3 was the weakest inhibitor for ^{125}I-ET-1 binding to the outer part of the retina, and the area anatomically corresponding to the choroid and the sclera.

^{125}I-ET-1 binding characteristics were examined in a cold ligand-saturation study using rat whole eye sections in the presence of a fixed amount of ^{125}I-ET-1 (32.6pM) and

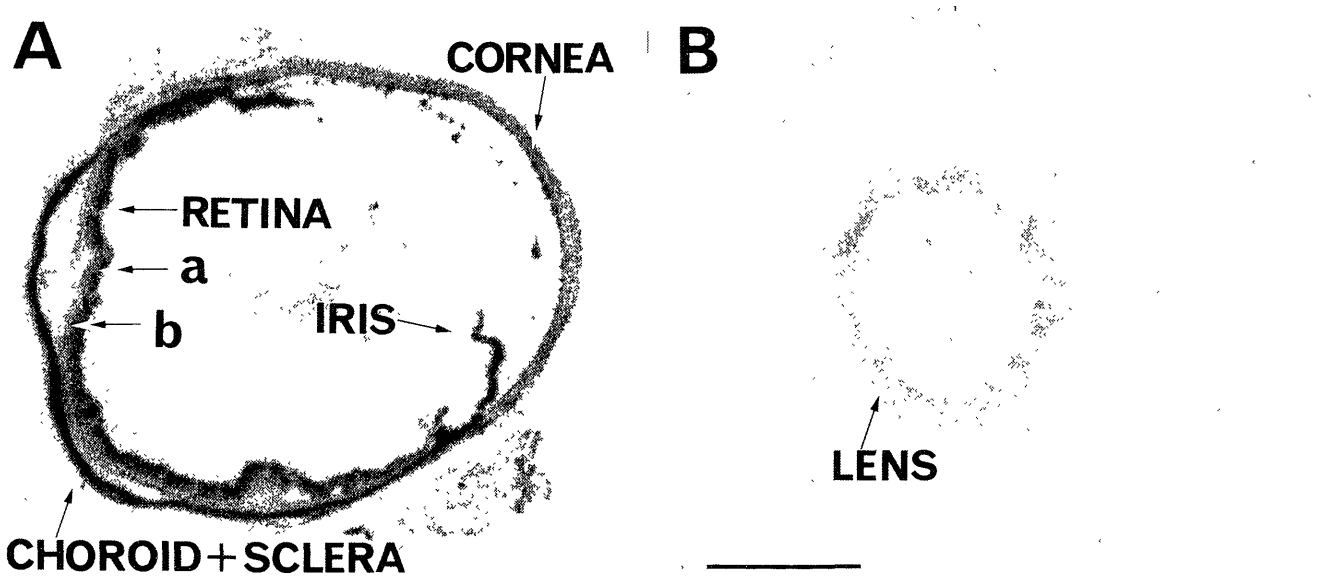

Fig. 3. Receptor autoradiographic localization of ^{125}I-endothelin-1 binding sites in the rat eye. Consecutive, 10-μm-thick sections were labeled with 32.6pM ^{125}I-ET-1 in the absence (total binding, A) or presence of 1.0μM ET-1 (non-specific binding, B), in vitro. After incubation dried sections were exposed to Hyperfilm-^{3}H for 2 days. RETINA (a), inner part of the retina; RETINA (b), outer part of the retina; TB, total binding. Bar = 2.0mm.

Table 1. Specific binding concentrations (SB) of ^{125}I-endothelin-1 (32.6pM) and binding concentrations in the presence of 10μM endothelin-3 (ET-3) in the rat eye. These binding concentrations (fmol/mg) were calculated by subtracting non-specific binding concentrations obtained at incubation with the radioligand in the presence of 1.0μM endothelin-1 (ET-1) from total binding concentrations. The ratio (%) of values of binding concentrations remained in the presence of ET-3 to SB was slso calculated (ET-3/SB). Values were expressed as means ± S. E. of three rats.

	cornea	iris	inner retina	outer retina	choroid + sclera
SB	16.5 ± 0.4^{a}	34.5 ± 3.6^{b}	28.3 ± 0.7^{c}	15.5 ± 11^{d}	31.0 ± 2.4^{e}
ET-3	0.9 ± 0.0	3.1 ± 0.4	4.1 ± 0.4	4.4 ± 0.1	6.9 ± 0.5
ET-3/SB	5.0 ± 0.3^{f}	6.8 ± 0 1^{g}	14.5 ± 2.7^{h}	22.0 ± 2.7^{i}	30.7 ± 3.1^{j}
ET-1	0.2 (± 0.03)	0.7(± 0.02)	0.8 (± 0.08)	0.2 (± 0.02)	0.7 (± 0.04)

Significant differences among the SB. P < 0.05 (a < b, F = 22.49), P < 0.01 (d < b, F = 25.16).
Significant differences among the ET-3/SB (%). P < 0.01 (f < g, F = 26.63; f < h, F = 57.34; f < i, F = 39.77; f < j, F = 67.38; g < h, F = 40.41; g < i, F = 32.45; g < j, F = 58.91; h < j, F = 23.64.

increasing concentrations of unlabeled ET-1, ranging from 1.0pM to 1.0μM (Fig. 4 and Table 2). ^{125}I-ET-1 binding to the eye sections was monophasically inhibited by unlabeled ET-1 with a high affinity, as evidenced by the straight line in the Scatchard plot. We used ET-3 as an inhibitor to characterize ^{125}I-ET-1 binding sites in the rat eye, since a comparison of the potencies of ET-1 and ET-3 at inhibiting ^{125}I-ET-1 binding discriminates between multiple endothelin receptors[6,7]. The inhibition curve obtained with ET-3 was bimodal. Scatchard analysis of the data obtained with the program LIGAND indicated the presence of a low-affinity site with an inhibition constant (K_i) of 7.06nM for ET-3, and a high affinity-site with a K_i of 53pM. Thus, the binding characteristics suggested that the receptors, ET_A and ET_B are present in the rat eye.

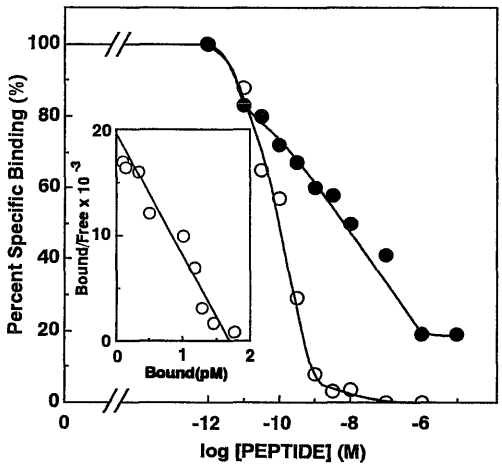

Fig. 4. Cold-ligand saturation and displacement experiments of ^{125}I-endothelin-1 (^{125}I-ET-1) binding to rat eye sections. Consecutive tissue sections were incubated with 32.6pM ^{125}I-ET-1 in the absence or presence of increasing concentrations of unlabeled ET-1 (○) or ET-3 (●). A typical Scatchard plot (inner part in the figure) was obtained by displacing the binding of 32.6pM ^{125}I-ET-1 by unlabeled ET-1 (○), by using the LIGAND computer program.

Table 2. Binding parameters obtained from cold-ligand saturation and displacement experiment done for endothelin-1 and endothelin-3, respectively; dissociation constant (K_d), maximum binding capacity (B_{max}), and inhibition constant (K_i) of specific ^{125}I-endothelin-1 binding sites in the rat eye. Values are expressed as means ± S. E. of three rats.

	K_d (pM)	B_{max} (fmol/mg)	K_i (pM) high	K_i (pM) low
rat whole eye	128 ± 11	189 ± 79	53 ± 10	7060 ± 2018

Data were analyzed using the program LIGAND.

Disscussion

ETs are biologically active peptides functioning within the eye and we investigated the localization and characterization of the specific ^{125}I-ET-1 binding sites. A comparison between localization of the receptors presented here and mRNAs encoding endothelin receptors reported by MacCumber *et al.* (1989)[5] seems pertinent to the functional significance of the ET family peptides within the eye. Accumulating evidence revealed that the ET family peptides function as mediators in autocrinal and paracrinal systems[6,7]. The receptors and the production system coexist in the iris, whereas the cornea and the choroid seem to possess only the receptors. Thus, a paracrinal system may function to regulate the pupil size in the iris[3,16,17,18], and an autocrinal system equipped with the ET_B receptor seems to operate in the cornea.

Based on the binding characteristics obtained in displacement studies, we postulate that the two endothelin receptors, the ET_A receptor[6], preferentially recognized by ET-1 with a much higher affinity than by ET-3, and the ET_B receptor[7], which is bound by ET-1, ET-2 and ET-3 with the same affinity, exist within the rat eye. Of particular interest was our observation that there was a difference between the potencies of 10μM ET-3 at inhibiting ^{125}I-ET-1 binding to different rat eye areas. As ET-3 almost fully inhibited specific binding to the cornea, it may be that the cornea mainly carries the ET_B receptor. Based on findings using in situ hybridization techniques, Hori *et al.*[19] reported that mRNA encoding the rat ET_B receptor is widely distributed in various endothelial cells and regulates water and electrolyte permeability in epithelial cells of the choroid plexus, ependymal cells lining the ventricle and renal endothelial cells of the glomerulus, the vasa recta bundle and thin segments of Henle's loop. Taken together with findings that ET-1 inhibited Na^+, K^+-ATPase in the tubular epithelial cells of the renal collecting duct[20] and cultured rat vascular smooth muscle cells[21], the possibility that ET family peptides play a function related to osmotic control between the anterior chamber and the corneal stroma by interacting with the ET_B receptor would have to be considered. As the astrocytic ET_B receptor has a function in proliferation of its own cells[22], our finding that the ET_B receptor is apparently present in the cornea serves to elucidate the regulatory roles of ET family peptides in the eye.

In areas such as the iris and the choroid, with their dense vascular components, we observed a lesser degree of potency of ET-3 in inhibiting ^{125}I-ET-1 binding. The ET_A receptor expressed in vascular smooth muscle cells[22] may contribute to inhibiting the potency of ET-3. There seems to be two subtypes of endothelin receptors in these areas, however, the presence and the proportion of the ET_A and ET_B receptors remains speculative until more is learned of ^{125}I-ET-1 binding characteristics.

The existence of specific ^{125}I-ET-1 binding sites in the retina was of particular interest. Although we did not observe the cellular localization of the endothelin receptors within part of the sensory retina, because of limitations in

the power of resolution of our technique, nevertheless, as neuronal cells such as rod bipolar, cone bipolar, bipolar and horizontal cells are present in the sensory retina, the finding of these receptors in this special area paves the way toward elucidation of the neuronal significance of ET family peptides.

We found in kinetic experiments carried as a function of incubation time that ^{125}I-ET-1 specifically bound to rat eye sections reached a maximum at 48hr and a plateau was maintained for up to 72hr at 4℃, under the conditions described. This slow association differs from findings obtained using partially purified membrane materials where ^{125}I-ET-1 reached a maximum within 3hr[23,24,25,26,27]. We added phosphoramidon[13], an inhibitor of endothelin metabolizing neutral endopeptidase (EC 3.4.24.11) to the incubation buffer to prevent the radioligand from degradation during a long incubation time. A similar slow association was obtained in the case of ^{125}I-ET-1 binding to rat brain sections done using receptor autoradiographic technique with incubation time of 24hr at 4 ℃[28].

In summary, we obtained evidence to support the idea that ET family peptides function in the rat eye as autocrinal and paracrinal transmitters. Although ET_A and ET_B receptors are apparently present in rat eyes, the precise heterogeneity and cellular localization of the receptors remain to be elucidated.

Acknowledgements

I am deeply indebted to Professors T. Amemiya and K. Taniyama, Departments of Opthalmology and Pharmacology 2, Nagasaki University School of Medicine for support. I also thank Drs. M. Niwa, A. Himeno, K. Shigematsu and S. Shibata for collaboration, Y. Hamada and K. Matsuo for technical assistance, and M. Ohara for pertinent comments.

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