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

Junko Tashiro

Department of Ophthalmology, Nagasaki University School of Medicine, 7-1 Sakamoto 1-chome, Nagasaki 852, Japan

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.

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°C, 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°C. Frozen, 10-μm-thick eye sections were cut in a cryostat at -20°C, thaw-mounted onto gelatin-coated slides, and stored overnight under vacuum at 4°C.

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°C) for 10min in the incubation buffer, consecutive tissue sections were incubated at 4°C 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 and 0.2% (w/v) bovine serum albumin (proteinase-free). Following these incubations, the slides were washed 3 times (1min each) at 4°C in 50mM Tris-HCl buffer (pH 7.4), 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$^{3+}$). These dried, labeled sections were exposed to radioluminographic imaging plates (Type BAS-III) with...
calibrated 10-μm-thick [125I]-standards ([125I] 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 run on each imaging plate. After quantitation, the sections labeled with the radioligand were also exposed to Hyperfilm-3H (Amersham) to acquire autoradiograms. The films were developed at 4°C 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°C for 30min, 1hr, 2hr, 6hr, 12hr, 24hr, 36hr, 48hr and 72hr, then the degradation of [125I]-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 C18 column (5μm C18, 3.9 x 150mm, Waters, U. S. A.). [125I]-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. 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°C with 26.7pM [125I]-ET-1, we found that specific [125I]-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 [125I]-ET-1 in the incubation buffer (Fig. 2). Hence, the following binding experiments were carried out at 4°C for 48hr.

![Fig. 1. Effect of incubation time on specific binding of [125I]-endothelin-1 ([125I]-ET-1) to rat eye sections. Each point represents the mean of four determinations. Related tissue sections were incubated at 4°C with 26.7pM [125I]-ET-1 in the absence or presence of 1.0μM unlabeled ET-1.](image1)

![Fig. 2. High-performance liquid chromatographic analysis of [125I]-endothelin-1 ([125I]-ET-1) before (A) and after (B) incubation of 72hr at 4°C. The sample was loaded on a reverse-phase µBondasphere C18 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.](image2)
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) and MacCumber et al. (1989). Apparent specific binding concentrations were calculated by subtracting non-specific binding concentrations obtained at incubation with 32.6pM $^{125}$I-ET-1 in the presence 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 remained 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 10μM ET-3. As shown in Table 1, interestingly, ET-3 most potently inhibited binding to the cornea. In this area, binding concentrations remained 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.

![Fig. 3](image-url). 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-²H for 2 days. RETINA (a), inner part of the retina; RETINA (b), outer part of the retina; TB, total binding. Bar = 2.0mm.

<table>
<thead>
<tr>
<th></th>
<th>cornea</th>
<th>iris</th>
<th>inner retina</th>
<th>outer retina</th>
<th>choroid + sclera</th>
</tr>
</thead>
<tbody>
<tr>
<td>SB</td>
<td>16.5 ± 0.4</td>
<td>34.5 ± 3.6</td>
<td>28.3 ± 0.7</td>
<td>15.5 ± 11</td>
<td>31.0 ± 2.4</td>
</tr>
<tr>
<td>ET-3</td>
<td>0.9 ± 0.0</td>
<td>3.1 ± 0.4</td>
<td>4.1 ± 0.4</td>
<td>4.4 ± 0.1</td>
<td>6.9 ± 0.5</td>
</tr>
<tr>
<td>ET-3/SB</td>
<td>5.0 ± 0.3</td>
<td>6.8 ± 0.1</td>
<td>14.5 ± 2.7</td>
<td>22.0 ± 2.7</td>
<td>30.7 ± 3.1</td>
</tr>
<tr>
<td>ET-1</td>
<td>0.2 (± 0.03)</td>
<td>0.7 (± 0.02)</td>
<td>0.8 (± 0.08)</td>
<td>0.2 (± 0.02)</td>
<td>0.7 (± 0.04)</td>
</tr>
</tbody>
</table>

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). 125I-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 125I-ET-1 binding sites in the rat eye, since a comparison of the potencies of ET-1 and ET-3 at inhibiting 125I-ET-1 binding discriminates between multiple endothelin receptors6,7. 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 (Ki) of 7.06nM for ET-3, and a high affinity-site with a Ki of 53pM. Thus, the binding characteristics suggested that the receptors, ETA and ETB are present in the rat eye.

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

<table>
<thead>
<tr>
<th></th>
<th>Kd (pM)</th>
<th>Bmax (fmol/mg)</th>
<th>Ki (pM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rat whole eye</td>
<td>128 ± 11</td>
<td>189 ± 79</td>
<td>53 ± 10</td>
</tr>
</tbody>
</table>

Data were analyzed using the program LIGAND.

Discussion

ETs are biologically active peptides functioning within the eye and we investigated the localization and character-
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 125I-ET-1 specifically bound to rat eye sections reached a maximum at 48hr and a plateau was maintained for up to 72hr at 4°C, under the conditions described. This slow association differs from findings obtained using partially purified membrane materials where 125I-ET-1 reached a maximum within 3hrs.

We added phosphoramidon, 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 125I-ET-1 binding to rat brain sections done using receptor autoradiographic technique with incubation time of 24hr at 4°C.

In summary, we obtained evidence to support the idea that ET family peptides function in the rat eye as autocrine and paracrine 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 Ophthalmology and Pharmacology 2, Nagasaki University School of Medicine for technical assistance, and M. Ohara for pertinent 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.

References