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Binding of a new bioactive 31-amino-acid endothelin-1 to an endothelin ET_B or ET_B-like receptor in porcine lungs

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Abstract

Endothelin-1-(1–31) is a new bioactive 31-amino-acid-length peptide generated from big endothelin-1 by chymase or other chymotrypsin-type proteases with various pathophysiologic functions. In this study, we have detected the specific and monophasic binding of [^{125}I]endothelin-1-(1–31) in porcine lung membranes. Competition studies of [^{125}I]endothelin-1-(1–31) binding by unlabeled endothelin-1-(1–31), endothelin-1, endothelin-3, and antagonists and agonists of endothelin ET_A and ET_B receptors suggest that the binding protein is an endothelin ET_B or ET_B-like receptor rather than an endothelin ET_A receptor in porcine lungs. Kinetic studies showed that the affinity of endothelin-1-(1–31) to its receptor was approximately one order of magnitude lower than that of endothelin-1, and that the specific binding of endothelin-1-(1–31) was about 19% of endothelin-1 binding. The binding of [^{125}I]endothelin-1-(1–31) was extremely slow, slower even than that of endothelin-1, and nearly irreversible. This unique quasi-irreversibility may explain the slow-onset and long-lasting biologic effects of this peptide in vivo.

Keywords: Endothelin-1; Endothelin-1-(1–31); Endothelin ET_A receptor; Endothelin ET_B receptor; Radio receptor binding assay; Lung, porcine

1. Introduction

The endothelins, a family of 21-residue peptides, were first isolated from the culture medium of porcine aortic endothelial cells, and shown to be vasoconstrictors (Yanagisawa et al., 1988; Yanagisawa and Masaki, 1989). Three forms of the peptides have been characterized, and designated as endothelin-1, -2 and -3. Recent studies have indicated that endothelins and their receptor subtypes are expressed in various cell types (Webb, 1998; Goldie et al., 1996), and have a variety of physiologic and pathologic functions, not only as smooth muscle constrictors (Doherty, 1992; Uchida et al., 1988), but also as inflammatory mediators (Michael and Markewitz, 1996; Sampaio et al., 2000).

The bioactive endothelin peptide family has expanded to include the new smooth muscle-constricting 31-amino-acid endothelin-1-(1–31), -2-(1–31) and -3-(1–31), generated from big endothelins through specific cleavage of the Tyr^{31}–Gly^{32} bond by human chymase or other chymotrypsin-type proteases (Nakano et al., 1997; Hanson et al., 1997). More recently, endothelin-1-(1–32) generated from big endothelin-1 through cleavage of the Gly^{32}–Leu^{33} bond by matrix metalloproteinase-2 has also been described (Fernandez-Patron et al., 2001). Analyses of the concentration of endothelins-(1–31) in human tissues with specific sandwich-type enzyme immunoassay have revealed that the three types of endothelins-(1–31) are distributed in human lungs (Okishima et al., 2001), and are present in human granulocytes in higher concentrations than 21-amino-acid-length endothelins (Okishima et al., 1999), suggesting that they have pivotal pathophysiologic functions in vivo. Endothelins-(1–31) have potent smooth muscle-constricting property (Nakano et al., 1997; Kido et al., 1998; Kishi et al., 1998), cell-proliferating activity (Yoshizumi et al., 1998b; Hayasaki-Kajiwara et al., 1999; Mazzocchi et al., 2000) and chemotactic activity toward mouse eosinophils in vivo (Sharmin et al., 2002), as well as toward human neutrophils and monocytes in vitro (Cui et al., 2001). These biologic activities of endothelins-(1–31) are not the consequence of conversion to the corresponding 21-amino-acid-length endothelins by phosphoramidon-sensitive endo-

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thelin-converting enzymes or metalloendopeptidases (Nakano et al., 1997; Yoshizumi et al., 1998a; Inui et al., 1999). The effects of specific endothelin ET_A receptor antagonists, BQ123 [cyclo-(D-Asp-Pro-D-Val-Leu-D-Trp),Na] and BQ485 (Hexahydro-1*H*-azepinylcarbonyl-Leu-D-Trp-D-Trp-OH,Na), and ET_B receptor antagonist, BQ788 [*N-cis*-2,6-Dimethylpiperidinocarbonyl-L-γ-MeLeu-D-Trp(MeOCO)-D-Nle-OH,Na] on the biological activities of endothelins-(1–31) vary depending on species, tissues and cell types (Cui et al., 2001; Mazzocchi et al., 2000; Kishi et al., 1998; Goldie et al., 1996). Despite these in vivo biologic observations, the binding characteristics of endothelins-(1–31) to their receptors have not been clarified.

In the present study, we analyzed the binding characteristics of [^{125}I]endothelin-1-(1–31), compared with [^{125}I]endothelin-1, toward human recombinant endothelin ET_A and ET_B receptors expressed in Chinese hamster ovary (CHO) cells and porcine lung membranes, which contain various endothelin receptors and are a model of the human lung endothelin system (Goldie et al., 1996). The results suggest that endothelin-1-(1–31) binds with extremely slow association kinetics to endothelin ET_B or ET_B-like receptors, but not the endothelin ET_A receptor in porcine lung membranes.

2. Materials and methods

2.1. Materials

Endothelin-1 and endothelin-1-(1–31) were purchased from Peptide Institute (Osaka, Japan) and chloramine T and sodium metabisulphite from Wako (Osaka, Japan). Endothelin ET_A receptor-specific antagonist, BQ123, and an endothelin ET_B receptor-specific antagonist, BQ788, were purchased from Calbiochem-Novabiochem (San Diego, CA). Endothelin ET_B receptor-specific agonist IRL1620 [Suc-(Glu^9,Ala^{11,15})-endothelin-1(8–21)] was purchased from Peptide Institute.

2.2. Radioiodination of endothelin-1 and endothelin-1-(1–31)

Endothelin-1 and endothelin-1-(1–31) were radioiodinated with [^{125}I]-NaI (Amersham Biosciences, Piscataway, NJ, USA) by the chloramine T method (Hunter and Greenwood, 1962). The radiolabeling was carried out in a molar ratio of 6:3:2 (peptide/iodine/chloramine T) in sodium phosphate buffer, at pH 7.5 and room temperature for 2 min. After the reaction, the labeled peptides were purified by reversed-phase high performance liquid chromatography on a TSK ODS-120T column (4.6 × 250 mm, Tosoh, Tokyo, Japan), with a linear gradient of 18–90% acetonitrile in 0.1% trifluoroacetic acid (Okishima et al., 1999). The specific radioactivities of both [^{125}I]endothelin-1 and [^{125}I]endothelin-1-(1–31) were in the range of 300–450 μCi/pmol. Radioactivity was measured by a Packard Cobra, Model 5002, gamma counter at a counting efficiency >72%. Concentrations of the labeled endothelins were determined with enzyme immunoassay kits from IBL (Fujioka, Japan) (Okishima et al., 1999) and the ligand depletion method. The concentrations measured by either method were nearly identical.

2.3. Preparation of porcine lung membranes

Porcine lung membranes were prepared by a slightly modified method described previously (Kozuka et al., 1991). The Animal Research Committee of the University of Tokushima School of Medicine approved the protocol of this study. Freshly prepared porcine lungs were minced with scissors and homogenized in a Waring blender for 2 min in 3 volumes of 20 mM Tris–HCl buffer, pH 7.4, containing 145 mM NaCl, 5 mM KCl, 3 mM $MgCl_2$, 0.2 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin and 1 μM pepstatin. The homogenates were centrifuged at 5000 × *g* for 20 min at 4 °C, and the supernatants were discarded. The membrane fractions of the precipitates were washed three times with the same buffer by suspension in 3 volumes of the buffer and recentrifuged. Finally, the protein concentrations of the membranes were measured by the bicinchonic acid protein assay reagent (Pierce, Rockford, IL, USA) using bovine serum albumin as a standard. The membranes were stored in appropriate concentrations at −80 °C until use. Membrane fractions of CHO-K1 cells stably expressing recombinant human endothelin ET_A or ET_B receptors were purchased from Euroscreen (Brussels, Belgium).

2.4. Radio receptor binding assay

Binding assays were performed using [^{125}I]endothelin-1 or [^{125}I]endothelin-1-(1–31). The binding reaction for the human recombinant endothelin ET_A and ET_B receptors in the membranes of CHO cells was performed according to the manufacturer's instructions in 50 mM HEPES buffer, pH 7.4, containing 1 mM $CaCl_2$, 5 mM $MgCl_2$, 0.1% NaN_3 and 0.5% bovine serum albumin. The binding reaction for the porcine lung membranes was carried out in 20 mM Tris–HCl buffer, pH 7.4, containing 145 mM NaCl, 5 mM KCl, 3 mM $MgCl_2$, 0.2 mM phenylmethanesulfonyl fluoride, 1 μM leupeptin, 1 μM pepstatin and 1 mg/ml bovine serum albumin as reported (Hagiwara et al., 1990). After the reaction, the membranes were precipitated by centrifugation at 20 000 × *g* for 20 min at 4 °C, washed gently with the binding buffer and recentrifuged for 15 min to separate free and bound [^{125}I]-ligands before counting the membrane-bound radioactivity. Nonspecific binding was defined in the presence of 1000-fold molar excess of unlabeled ligands. Since endothelin binding may not reach equilibrium under normal binding conditions due to the quasi-irreversible binding characteristics, thus special consideration should

be taken for analysis of the binding data (Waggoner et al., 1992). For the purpose of comparing our results with others, while somewhat problematic, we have determined the dissociation constant (K_d) and the maximum binding sites (B_{max}) by Scatchard analysis at the near equilibrium binding conditions similar to that used by other laboratories as described below (Chiou et al., 1997; Hilal-Dandan et al., 1997). Although [^{125}I]endothelin-1 binding toward both subtypes of endothelin receptors in porcine lung membranes at 25 °C reached a near plateau level after incubation for 1.5 h as previously reported (Chiou et al., 1997), [^{125}I]endothelin-1(1–31) binding slowly reached a near plateau level after 2 h of incubation. In addition, little dissociation of [^{125}I]endothelin-1-(1–31) already bound on porcine lung membranes by a 5000-fold excess of unlabeled ligand was observed, as previously reported with [^{125}I]endothelin-1 (Chiou et al., 1997). From these results, incubation times for the saturation and competition experiments were chosen as follows: 4 and 2 h for saturation and competition studies of [^{125}I]endothelin-1 binding, respectively, and 5 and 3 h for saturation and competition studies of [^{125}I]endothelin-1-(1–31) binding, respectively, since the equilibrium process of binding of endothelin-1-(1–31) was slower than that of endothelin-1.

2.5. Data analysis

All experiments were performed at least four times, each in duplicate. Nonlinear regression analyses of saturation of binding, competition of binding, and time courses for association and dissociation of binding were performed using PRISM Version 3.0 (Graphpad Software, San Diego, CA, USA). The data from saturation of binding on recombinant endothelin receptors were fitted to a single site hyperbolic function to determine K_d and B_{max} values. These K_d values and the IC_{50} values obtained from competition experiments were used in the calculation of inhibition constant (K_i) values by Cheng and Prusoff's (1973) equation. In the studies of endothelins binding to porcine lung membranes, we compared the statistical fitness of two models using F-test, i.e., one-site or two-site binding models. Results were considered significant when P was <0.05.

3. Results

3.1. Endothelin-1 competes strongly, and endothelin-1-(1–31) weakly, with the binding of [^{125}I]endothelin-1 to human endothelin ET_A and ET_B receptors

To compare the binding characteristics of endothelin-1-(1–31) to human endothelin ET_A and ET_B receptors with those of endothelin-1, we used the membrane fractions of CHO cells expressing human ET_A and ET_B receptors. [^{125}I]Endothelin-1, as expected, specifically bound with a monophasic binding isotherm to the CHO cell membranes expressing ET_A or ET_B receptors. Nonspecific binding was approximately 25% of the total binding. Specific binding of [^{125}I]endothelin-1-(1–31) to those human recombinant receptors, however, was little detected. Specific binding of [^{125}I]endothelin-1 to human recombinant endothelin ET_B receptors in comparison with binding of [^{125}I]endothelin-1-(1–31) was shown in Fig. 1. Similar binding characteristics of [^{125}I]endothelin-1 and [^{125}I]endothelin-1-(1–31) to human endothelin ET_A receptors were observed (data not shown). The K_d and B_{max} binding constants of [^{125}I]endothelin-1 were 95 ± 18 pM and 50.3 ± 3.4 pmol/mg protein, respectively, for human endothelin ET_A receptors, and 105 ± 26 pM and 7.1 ± 0.6 pmol/mg protein, respectively, for human endothelin ET_B receptors. These values are consistent with previously reported results (Elshourbagy et al., 1993).

We further analyzed the competition of [^{125}I]endothelin-1 (100 pM) binding to the human recombinant ET_A and ET_B receptors by increasing concentrations of endothelin-1, endothelin-3, the selective endothelin ET_A receptor antagonists, BQ123 and BQ485, the endothelin ET_B receptor antagonist, BQ788, and endothelin-1-(1–31) (Fig. 2A and B, respectively). As expected, [^{125}I]endothelin-1 binding to endothelin ET_A receptor was predominantly inhibited by unlabeled endothelin-1, moderately by BQ123 and weakly by endothelin-3 and BQ788. The magnitude of competition by endothelin-3 and BQ788 was approximately three orders lower than by endothelin-1. The magnitude of competition by endothelin-1-(1–31), however, was the lowest among the compounds tested, approximately five orders magnitude lower than by endothelin-1, indicating little binding of endothelin-1-(1–31) to the human ET_A receptor.

[^{125}I]Endothelin-1 binding to endothelin ET_B receptor in the membranes of CHO cells was competed predominantly

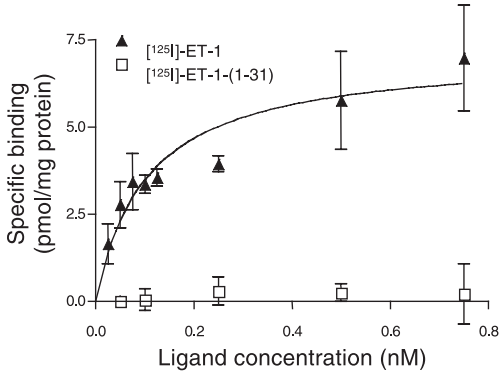

Fig. 1. Equilibrium binding of [^{125}I]endothelin-1 and [^{125}I]endothelin-1-(1–31) to human endothelin ET_B receptors expressed in the membranes of CHO cells. Increasing concentrations of [^{125}I]endothelin-1 (▲) and [^{125}I]endothelin-1-(1–31) (□) were incubated with the membranes (0.175 ng) for 4 h at 25 °C. Nonspecific binding was determined in the presence of 1000-fold higher concentrations of unlabeled endothelins at each point. Specific binding was calculated by subtracting nonspecific binding from total binding. Each value represents the mean ± S.E. of three separate experiments.

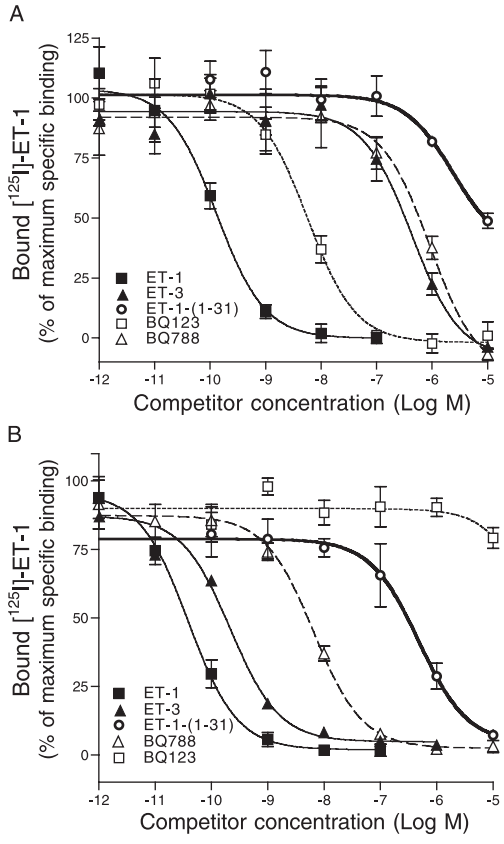

Fig. 2. Competition of [^{125}I]endothelin-1 binding by various endothelin derivatives and receptor antagonists to endothelin ET_A receptor (A) and ET_B receptor (B) expressed in the membranes of CHO cells. [^{125}I]Endothelin-1 (100 pM) was incubated for 2 h at 25 °C with increasing concentrations of unlabeled endothelin-1 (■), -3 (▲), ET_A receptor antagonist BQ123 (□), ET_B receptor antagonist BQ788 (△), and endothelin-1-(1–31) (○). The membrane proteins of endothelin ET_A receptor (20 ng) and ET_B receptor (175 ng) were used for each assay. Each value represents the mean ± S.E. of four separate experiments. ET-1 = endothelin-1.

by unlabeled endothelin-1 and endothelin-3, moderately by BQ788 and weakly by endothelin-1-(1–31). IRL1620 peptide, a selective endothelin ET_B receptor agonist, competed in a manner similar to BQ788 (data not shown). Nearly no competition was observed by BQ123. The efficiency of competition of binding by endothelin-1-(1–31) was approximately four orders of magnitude lower than by endothelin-1. These results indicate that the human recombinant endothelin ET_A and ET_B receptors are not the binding proteins for endothelin-1-(1–31), although the human endothelin ET_B receptor in CHO cells interacted weakly with endothelin-1-(1–31), with a K_i value of 235 nM.

3.2. Characterization of [^{125}I]endothelin-1-(1–31) binding to porcine lung membranes

We next analyzed the binding of [^{125}I]endothelin-1-(1–31) to porcine lung membranes and compared the binding with that of [^{125}I]endothelin-1. Saturation binding of [^{125}I]endothelin-1 in porcine lung membranes yielded a B_{max} value of 4.9 ± 1.2 pmol/mg protein, and approximately 30% and 70% of the specific binding was prevented by 1 μM of BQ123 and BQ788, respectively. These results are consistent with previous reports (Elshourbagy et al., 1993; Chiou et al., 1997), and are supported by the results of quantitative autoradiographic studies (Goldie et al., 1996).

Specific binding of [^{125}I]endothelin-1-(1–31) to porcine lung membranes was observed and the binding was saturable as shown in Fig. 3. The Scatchard transformation of the data shows a single class binding site for endothelin-1-(1–31) (inset in Fig. 3). The apparent K_d value for endothelin-1-(1–31) was 856 ± 220 pM, and B_{max} was 0.92 ± 0.11 pmol/mg protein. In comparison with the saturation binding of [^{125}I]endothelin-1 to porcine lung membranes, the K_d value for [^{125}I]endothelin-1-(1–31) indicates that its affinity for the receptor is approximately one order of magnitude lower than that of endothelin-1, and its B_{max} value corresponding to approximately 19% of the number of [^{125}I]endothelin-1 binding sites.

The competition of [^{125}I]endothelin-1-(1–31) binding to the membranes of porcine lungs by unlabeled endothelin-1-(1–31), endothelin-1, endothelin-3, BQ123 and BQ788 is shown in Fig. 4. The competition by endothelin-1-(1–31) was approximately one order of magnitude lower than by endothelin-1 and -3, and approximately 2.7 times higher than by BQ788. Nearly no competition by the other endothelin ET_A receptor antagonists, BQ123 and BQ485, was observed. IRL1620, an endothelin ET_B receptor agonist, also prevented effectively the binding of [^{125}I]endothelin-1-(1–31). The K_i values of these compounds are shown in Table 1. The competition of binding of [^{125}I]endothelin-1-(1–31) to porcine lungs by endothelin-1, endothelin-3, BQ788 and BQ123, except endothelin-1-(1–31), were similar to the competition of binding of [^{125}I]endothelin-1 to

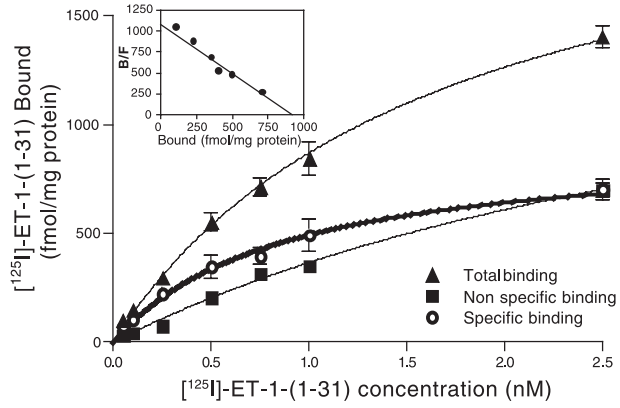

Fig. 3. Saturation curves for [^{125}I]endothelin-1-(1–31) binding to porcine lung membranes. [^{125}I]Endothelin-1-(1–31) was incubated with 10 μg of membranes of porcine lungs for 5 h at 25 °C. Nonspecific binding was determined by incubation with 1000-fold higher concentrations of unlabeled endothelin-1-(1–31) at each point. Scatchard transformation of specific binding data is shown in the inset. Each value represents the mean ± S.E. of four separate experiments. ET-1-(1–31) = endothelin-1-(1–31).

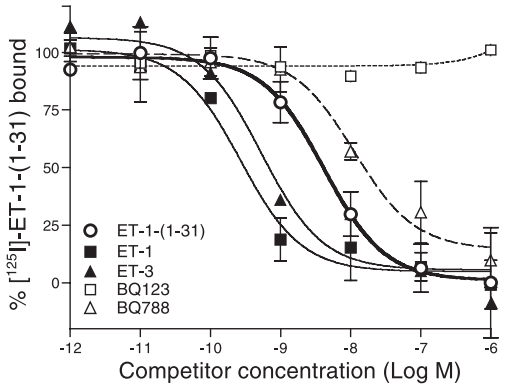

Fig. 4. Competition of [^{125}I]endothelin-1-(1–31) binding by various endothelin derivatives and endothelin receptor antagonists in the membranes of porcine lungs. Binding of [^{125}I]endothelin-1-(1–31) (500 pM) to porcine lung membranes was competed by increasing concentrations of endothelin-1 (■), endothelin-3 (▲), endothelin-1-(1–31) (○), BQ123 (□) and BQ788 (△). The experiments were carried out for 3 h at 25 °C. Each value represents the mean ± S.E. of four separate experiments. ET-1-(1–31) = endothelin-1-(1–31).

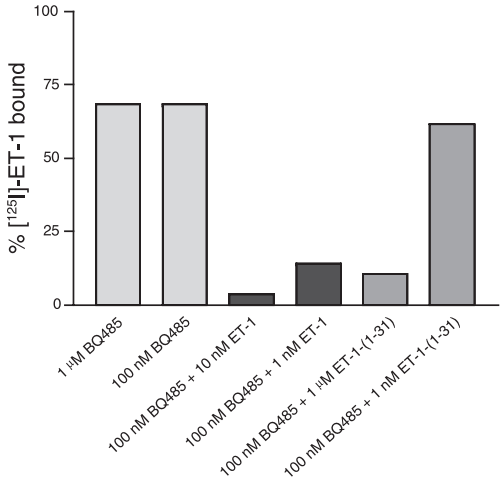

Fig. 5. Selective inhibition of [^{125}I]endothelin-1 binding to endothelin ET_B receptors in porcine lung membranes by endothelin-1 and endothelin-1-(1–31). Competition of [^{125}I]endothelin-1 binding by unlabeled endothelin-1 and endothelin-1-(1–31) was measured in the presence of 1 μM BQ485 as described under Materials and methods. Nonspecific binding was measured in the presence of 1 μM BQ485 and 1 μM unlabeled endothelin-1. Each result represents percentages of specific binding of [^{125}I]endothelin-1 in at least three independent experiments.

human recombinant endothelin ET_B receptor, illustrated in Fig. 2. Together, these results suggest that, in porcine lungs, the binding protein of endothelin-1-(1–31) is an ET_B or ET_B-like receptor, though not identical to the human ET_B receptor, since the efficiency of the competition by endothelin-1-(1–31) was significantly different in porcine lungs versus the human recombinant endothelin ET_B receptor.

Competition of selective binding of [^{125}I]endothelin-1 to endothelin ET_B or endothelin ET_B-like receptor in porcine lungs by endothelin-1 and endothelin-1-(1–31) was also analyzed under blocking the endothelin ET_A receptor by 100 nM of BQ485 (Fig. 5). Although endothelin-1 effectively competed the binding in the ranges at nM, endothelin-1-(1–31) competed a little at 1 nM and significant competition was observed at 1 μM. These results were consistent with those in Fig. 4.

Time course studies of [^{125}I]endothelin-1-(1–31) binding to porcine lung membranes are shown in Fig. 6. The binding slowly reached a nearly steady-state level by 2–3 h, and remained constant for up to 4 h thereafter, although [^{125}I]endothelin-1 binding reached nearly steady-state by 1–1.5 h under the same conditions (data not shown). Furthermore, nearly no dissociation of the bound [^{125}I]endothelin-1-(1–31) was observed when a 5000-fold excess of unlabeled ligand was added after incubation of the labeled ligand for 2 h. The apparent irreversibility of [^{125}I]endothelin-1-(1–31) binding was similar to that observed with the binding of [^{125}I]endothelin-1 to its receptor (Hilal-Dandan et al., 1997).

Table 1
K_i values of endothelin derivatives and endothelin receptor antagonists and agonist in the competition of [^{125}I]endothelin-1-(1–31) binding to the membranes of porcine lungs

Ligand	ET-1	ET-3	ET-1-(1–31)	BQ123	BQ485	IRL1620	BQ788
K_i (nM)	0.12	0.25	1.93	N.D.	N.D.	0.16	5.20

K_i values were determined by the Cheng and Prusoff's (1973) equation based on the data of IC_{50} and K_d values in Fig. 4.
ET-1 = endothelin-1; ET-3 = endothelin-3; N.D. = not determined because the competition was too weak and the data could not be fitted by the equation of competition binding curve.

3.3. Effect of protease inhibitor phosphoramidon on the binding of [^{125}I]endothelin-1-(1–31)

The possibility that binding of endothelin-1-(1–31) is the consequence of a conversion to endothelin-1 by endothelin-converting enzymes was examined by studying the effects

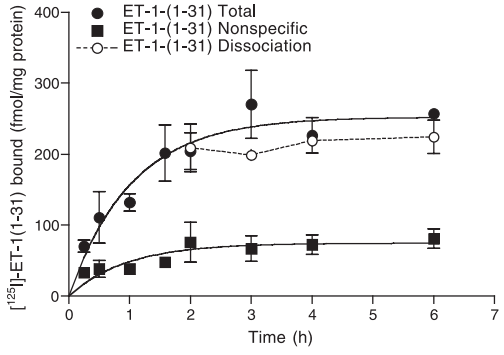

Fig. 6. Time course of [^{125}I]endothelin-1-(1–31) binding to the membranes of porcine lungs and dissociation of the bound ligand by the addition of excess unlabeled ligand. Porcine lung membranes (10 μg) were incubated with 200 pM of [^{125}I]endothelin-1-(1–31) at indicated periods of time at 25 °C (●). The dissociation of the binding was analyzed by the addition of a 5000-fold excess of unlabeled ligand after incubation for 2 h (○). See text for description of nonspecific binding (■) measurements. Each value represents the mean ± S.E. of four separate experiments. ET-1-(1–31) = endothelin-1-(1–31).

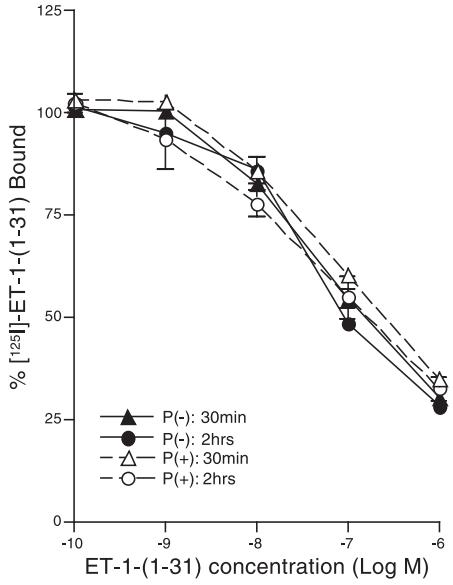

Fig. 7. Competition of [^{125}I]endothelin-1-(1–31) binding to the porcine lung membranes by unlabeled ligand in the presence versus absence of phosphoramidon. [^{125}I] Endothelin 1-(1–31) (500 pM) was incubated with porcine lung membranes with increasing concentrations of unlabeled endothelin-1-(1–31) in the presence or absence of 1 mM phosphoramidon for 30 min and 2 h. Data represent percentages of binding in the absence of unlabeled ligand. No significant difference in the rates of competition in the absence (▲, ●) versus presence (△, ○) of phosphoramidon (1 mM) for incubation periods of 30 min (△, ▲) and 2 h (○, ●). Each value represents the mean ± S.E. of four separate experiments. ET-1-(1-31) = endothelin-1-(1–31).

of phosphoramidon, an inhibitor of the enzymes, on the binding of endothelin-1-(1–31) to its receptor (Fig. 7). Competition profiles of binding of radiolabeled ligand to porcine lung membranes with increasing concentrations of unlabeled endothelin-1-(1–31) were similar in the presence or absence of 1 mM phosphoramidon for 0.5 and 2 h. Phosphoramidon, a nonselective metalloprotease inhibitor, inhibits the endothelin-converting enzymes 1 and 2 at IC_{50} values of 0.35–8.00 μM (Takahashi et al., 1993; Schmidt et al., 1994) and 4 nM (Emoto and Yanagisawa, 1995), respectively, and neutral endopeptidase at a K_i value of 2 nM (Kenny, 1977). These results indicate that binding of endothelin-1-(1–31) to its receptor is not a consequence of its conversion to endothelin-1.

4. Discussion

We have previously identified new, smooth muscle-constricting 31-amino-acid endothelins-(1–31), generated from big endothelins through specific cleavage by human mast cell chymase or other chymotrypsin-type proteases in various cells. These endothelins-(1–31) are found in human lungs, as well as major bioactive endothelin derivatives in human granulocytes. Although we have reported various pharmacologic effects of endothelins-(1–31) (Nakano et al., 1997; Kishi et al., 1998; Yoshizumi et al., 1998a; Cui et al., 2001; Sharmin et al., 2002), the characteristics of their receptor binding have not been clarified. Two subtypes of endothelin receptor, ET_A and ET_B (or ET_{B1}), have been described, and an additional receptor, ET_{B2}, has also been evoked (Hay et al., 1998; Bax and Saxena, 1994). The concentrations and distributions of these receptor subtypes vary among species and tissue types (Goldie et al., 1996), and this variability complicates the understanding of the biological effects of endothelins and endothelin receptor antagonists in vivo.

The biologic effects of endothelin-1-(1–31) and their inhibition by endothelin receptor antagonists also vary among species and tissue types. The biologic effects of endothelin-1-(1–31) are inhibited by the endothelin ET_A receptor antagonist in human neutrophils and monocytes, murine eosinophils, human coronary artery smooth muscle, and rat zona glomerulosa cells (Cui et al., 2001; Sharmin et al., 2002; Yoshizumi et al., 1998a; Mazzocchi et al., 2000). In contrast, in the rat aorta, the effects of endothelin-1-(1–31) are prominently inhibited by the endothelin ET_B receptor antagonist (Kishi et al., 1998). Recently, autoradiographic detection studies of [^{125}I]endothelin-1-(1–31) binding in the airways of rats revealed that it binds to both endothelin ET_A and ET_B receptors (Goldie et al., 2000). However, the receptor binding characteristics for endothelin-1-(1–31) have not been described.

We first characterized the receptor binding of endothelin-1-(1–31) in the membranes of porcine lungs, where the ET_A/ET_B receptors ratio is approximately 30:70 (Goldie et al., 1996). Specific and saturable binding of [^{125}I]endothelin-1-(1–31) with monophasic kinetics was observed in porcine lungs. Studies on the competition of [^{125}I]endothelin-1-(1–31) binding by unlabeled endothelin-1-(1–31), endothelin-1, endothelin-3, BQ123 and BQ788 suggested that the binding protein of endothelin-1-(1–31) is an endothelin ET_B or ET_B-like receptor, rather than an endothelin ET_A receptor (Figs. 4 and 5). Furthermore, differences between CHO cells expressing human recombinant endothelin ET_B receptor (Fig. 2B) and porcine lungs (Fig. 4) in the efficiency of competition of [^{125}I]endothelin-1-(1–31) binding by unlabeled endothelin-1-(1–31) suggest that the binding protein of endothelin-1-(1–31) in porcine lungs is not identical to the human endothelin ET_B receptor. In addition, competition studies indicated that the affinity of endothelin-1-(1–31) to its receptor was approximately one order of magnitude lower than that of endothelin-1. The proportion of specific binding sites for [^{125}I]endothelin-1-(1–31) in porcine lung membranes was approximately 19% of the [^{125}I]endothelin-1 binding sites. A difference in the number of binding sites between [^{125}I]endothelin-1 and [^{125}I]endothelin-1-(1–31) suggests that the porcine lungs contain sub-subtypes of ET_B receptor, which bind both endothelin-1 and endothelin-1-(1–31), although the affinity for endothelin-1-(1–31) is lower than that for endothelin-1. Despite the histological demonstration of [^{125}I]endothelin-1-

(1–31) binding to endothelin ET_A and ET_B receptors in rat airways (Goldie et al., 2000), we found only an ET_B or ET_B-like receptor for the binding of [^{125}I]endothelin-1-(1–31) in porcine lungs. However, the inhibitory effects of BQ123 on various biologic activities of endothelin-1-(1–31) (Cui et al., 2001; Sharmin et al., 2002; Yoshizumi et al., 1998a; Mazzocchi et al., 2000) suggest the existence of one or more additional endothelin ET_A or ET_A-like binding protein(s) for endothelin-1-(1–31) in other tissues.

The irreversibility of endothelin-1-(1–31) binding to its receptor(s) is a noteworthy characteristic. This unique property has previously been observed with the binding of endothelin-1 to its receptors, ET_A and ET_B, while binding of artificial endothelin analogs is mostly reversible and easy to dissociate (Chiou et al., 1997). The kinetic studies shown in Fig. 6 revealed that the association of endothelin-1-(1–31) is slower than that of endothelin-1 and its binding harder to dissociate. This extremely slow association and irreversibility of endothelin-1-(1–31) binding may explain the slower development of more prolonged contraction induced by endothelin-1-(1–31) in isolated porcine coronary arteries than by endothelin-1 (Kishi et al., 1998). It may also explain other observations, such as the durable eosinophil migration evoked by this peptide (Sharmin et al., 2002). In addition, endothelin-1-(1–31) may contribute to the prolonged increase in bronchial tone in obstructive airway diseases, such as asthma, as has been reported with endothelin-1 (Henry and Goldie, 1995). Furthermore, the long-lasting effects of this peptide are explained by its stability against proteases, such as endothelin-converting enzymes, which selectively recognizes the C-terminal region of big endothelin-1, thus does not hydrolyze endothelin-1-(1–31) (Okada et al., 1991).

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