Vol. 162, No. 1, 1989 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 14, 1989 Pages 130-137

IDENTIFICATION AND CHARACTERIZATION OF ENDOTHELIN BINDING SITES IN RAT RENAL PAPILLARY AND GLOMERULAR MEMBRANES

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Received May 18, 1989

SUMMARY The present study was designed to identify and characterize specific endothelin binding sites in membranes of rat renal papillae and glomeruli which appear to be target tissues for this new peptide hormone. Saturation binding studies indicate that the sites have a high and uniform affinity. The dissociation constants averaged 662 ± 151 and 1309 ± 123 pM and the receptor densities 7666 ± 920 and 5831 ± 348 fmol/mg protein for papillary and glomerular membranes, respectively. Endothelin 1, endothelin 3 and sarafotoxin all inhibited [^{125}I]-endothelin binding with IC_{50}' s in the 100-300 pM range, whereas unrelated peptides, namely angiotensin II, atrial natriuretic peptide, and platelet-derived growth factor failed to compete for [^{125}I]-endothelin binding. Deletion of the carboxyterminal tryptophan in endothelin 1 reduced its affinity for glomerular binding sites by 2 orders of magnitude. Specific endothelin binding to these membranes was maximal at pH 4 and was markedly inhibited as the pH was raised above 8. When [^{125}I]-endothelin is covalently linked to glomerular membrane binding sites, SDS-PAGE of these solubilized membranes followed by autoradiography reveals a predominant specifically labeled band of 45 kDa. Whether this band represents a subunit of the endothelin receptor(s), the receptor proper, or an intracellular endothelin binding protein remains to be determined.

Vascular endothelial cells secrete a 21-amino acid vasoactive peptide termed endothelin (1). Characterized by two intrachain disulfide bridges, it has a tertiary structure particularly homologous with alpha scorpiotoxins and the snake venom sarafotoxin (1-5). Sarafotoxin is a known coronary vasoconstrictor which binds to rat atrial and brain membranes leading to the hydrolysis of phosphoinositides (5). Endothelin, which also induces phosphoinositide hydrolysis (6, 7), competitively inhibits sarafotoxin binding to rat atrial and brain membranes, suggesting that these peptides share a common binding site and mechanism of action (8).

When injected as an intravenous bolus in rats, endothelin produces a sustained rise in mean arterial pressure due to a profound increase in peripheral vascular resistance. With regard to renal function, endothelin increases the glomerular filtration fraction and reduces glomerular filtration rate (9). Furthermore, in rabbit papillary collecting duct cells, endothelin inhibits ouabain-sensitive Na^+ K^+ ATPase via a prostaglandin-dependent process (10). Autoradiographs of [^{125}I]-endothelin binding to rat kidney shows the highest concentration of binding sites to be in the glomeruli and papilla (11).

High-affinity specific binding sites for [^{125}I]-endothelin have been described in vascular smooth muscle cells (6, 12-14), adrenal zona glomerulosa cells (15) and neural glia cells (17). Although Scatchard analysis suggests that these binding sites are of uniform affinity, the

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possibility exists that there are multiple receptor subtypes. Because renal glomeruli and papillary collecting ducts appear to be target tissues for endothelin, we sought to identify and characterize specific endothelin binding sites in membranes of these tissues. The binding sites were found to be of high and uniform affinity. When [^{125}I]-endothelin is covalently linked to glomerular membrane binding sites and these membranes subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), several specifically labeled binding sites appear on the autoradiogram, suggesting that multiple specific binding sites for endothelin are expressed in these tissues.

MATERIALS AND METHODS

Animal Preparation. Male Sprague-Dawley rats (Charles River Breeding Laboratories, Wilmington, MA) weighing 275-300 g were maintained on a standard rat chow (Ralston Purina Co., St. Louis, MO).

Tissue Preparation. Glomeruli were isolated as described previously (16). Glomeruli and papillae were homogenized in 4 volumes of ice-cold 0.25 M sucrose with a Polytron (Brinkman Instruments Co., Westbury, N.Y.) at setting number 5 with three 30-second bursts separated by 30-second cooling intervals. The volume of the homogenate was adjusted to 15 ml with 0.25 M sucrose and centrifuged at 1,000 G to remove unbroken cells and nuclei. The 1,000 G supernatant was then centrifuged at 40,000 G for 60 minutes and the resulting pellet resuspended in Hank's balanced salt solution (HBSS) (in mM: NaCl, 137; KCl, 5.4; KH_2PO_4, 0.44; Na_2HPO_4, 0.33; $MgSO_4$, 0.40; $MgCl_2$, 0.50; $CaCl_2$, 1.25; and $NaHCO_3$, 4.0; at pH 7.4) containing Hepes 25 mM. Membranes were stored in aliquots at -70^{o}C.

Receptor Binding Studies. The [^{125}I]-labeled porcine endothelin 1 and the homologous unlabeled peptide (Peninsula Laboratories, Inc., Belmont, CA) were used for radioreceptor studies. The crude microsomes were diluted in binding assay buffer of HBSS containing bovine serum albumin (BSA) 0.20 g/dl; Hepes 25 mM; bacitracin 1 mM; and phenylmethylsulfonyl fluoride 1 mM. Membranes were incubated with 20 to 40 pmol/liter of [^{125}I]-endothelin (specific activity 1200 Ci/mmole) in the presence or absence of unlabeled hormone under conditions as described below. Bound radioactivity was separated from free by filtration through glass fiber filters (Whatman GF/C), pretreated with 4 % BSA. Filter-associated radioactivity was counted in an LKB Clinigamma counter with 80 % efficiency. Nonspecific binding was defined as filter-associated radioactivity in the presence of 100 nM endothelin. Pilot studies showed that higher concentrations of endothelin did not further inhibit binding. Specific binding was the difference between total binding and nonspecific binding. Nonspecific binding was always < 1.5 % of the total radioactivity added. Saturation binding studies were performed by incubating membranes with increasing concentrations of [^{125}I]-endothelin (12 to 880 pM) until equilibrium at 25^{o}C. For competition binding inhibition studies, concentrations of radiolabeled endothelin ranging from 20 to 40 pmol/liter were used. Concentrations of unlabeled endothelin ranged from 0.01 to 100 nM. The reaction was initiated by the addition of 50 µl of either glomerular or papillary membranes (in a total volume of 150 µl). Increasing concentrations of various analogues, including porcine preproendothelin 110-130 amide (endothelin-like peptide) (1), endothelin 3, sarafotoxin S6b, and a tryptophan-deleted endothelin, as well as other peptide hormones, including angiotensin II, platelet-derived growth factor, atrial natriuretic peptide, were also assessed for their ability to compete for [^{125}I]-endothelin binding. Binding parameters were calculated by Scatchard analysis of specific binding data obtained from equilibrium saturation binding curves. The equilibrium dissociation constant, K_d, and the receptor density, R_o, were derived by linear regression of each of these Scatchard plots.

Covalent Labeling of Endothelin Binding Sites. Isolated glomeruli, glomerular and papillary membranes were incubated in binding assay buffer, as above with the addition of 1 mM benzamidine and 5 mM iodoacetamide, for 24 hours at 4^{o}C, with 400 pM [^{125}I]-endothelin, in the presence and absence of 100 nM endothelin. The covalent cross-linking agent *Bis* (sulfosuccinimidyl) suberate (Pierce Chemical Co., Rockford, IL) was then added to a final concentration of 0.1 to 1 mM, and incubation was continued for a further 60 minutes at 4^{o}C. The cross-linking reaction was stopped by the addition of an equal volume of a quench buffer containing 400 mM EDTA, 1 M tris(hydroxymethyl)aminomethane (Tris)·HCl, pH 6.8.

Membranes were then centrifuged at 13,000 G for 20 minutes to separate unbound hormone, and the resulting pellet was resuspended in 100 µl of sample buffer for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), containing 62.5 mM Tris base, 5 % SDS, 5 % glycerol, 0.01 % bromophenol blue, 2 % ß-mercaptoethanol, 5 mM EDTA, pH 6.8, and boiled for 3 minutes. The solubilized membranes were again subjected to centrifugation at 13,000 G to remove particulate matter followed by electrophoresis on a 7.5 % unidimensional polyacrylamide slab gel, and stained with Coomassie brilliant blue (Bio-Rad, Rockville, N.Y.). Autoradiography was performed by exposing the dried gel to a Kodak XAR 5 film at -20°C for 3 to 6 weeks. Identification of the molecular masses of the labeled bands was done by the use of reference standards (Bio-Rad) on the Coomassie blue-stained gel. A linear relationship between the relative mobility (R_f) and the log relative molecular mass of the standards was constructed and the unknown values calculated.

RESULTS

At 25°C, [^{125}I]-endothelin binding to papillary and glomerular membranes increased in a time-dependent manner and reached equilibrium at 4 hours. Binding remained stable for an additional 2 hours (data not shown). Binding increased linearly with protein concentration over a range of 0 to 10 and 0 to 7 mg/dl for papillary and glomerular membranes, respectively (data not shown). Only concentrations of membranes in this range were used for receptor binding studies.

Saturation studies of [^{125}I]-endothelin binding to papillary and glomerular membranes are shown in Figure 1 and 2. Binding approached saturation (Figures 1A and 2A) and Scatchard plots of the binding data (Figures 1B and 2B) were linear, indicating a uniform affinity for the binding sites. The mean equilibrium dissociation constants, K_d, determined from Scatchard analysis averaged 662 ± 151 and 1309 ± 123 pM and receptor densities, R_o, averaged 7666 ± 920 and 5831 ± 348 fmol/mg protein for papillary and glomerular membranes, respectively.

Figure 3 graphically summarizes the results of competitive binding inhibition studies for glomerular membranes. Unlabeled endothelin inhibited specific binding of the labeled ligand in a concentration-dependent manner and displaced 50 % of the labeled ligand (IC_{50}) at 140 ± 22 and

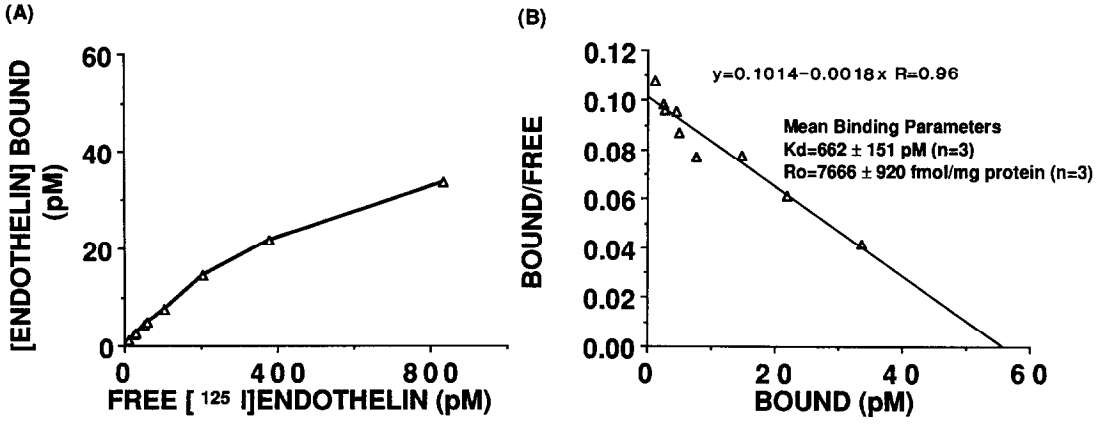


Figure 1. Representative saturation curve (A) and Scatchard plot (B) of specific [^{125}I]-endothelin binding to papillary membranes. Membranes were incubated with increasing concentrations of [^{125}I]-endothelin in the absence and presence of 100 nM unlabeled endothelin to equilibrium. Data points represent means of duplicate determinations. The means for receptor density (R_o) and equilibrium dissociation constant (K_d) derived from three separate experiments are shown.

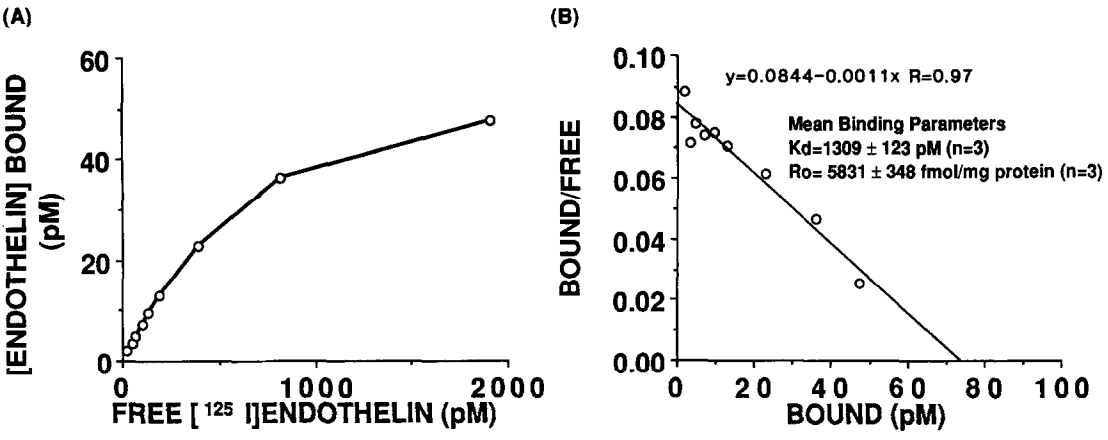


Figure 2. Representative saturation curve (A) and Scatchard plot (B) of specific [^{125}I]-endothelin binding to glomerular membranes. Membranes were incubated with increasing concentrations of [^{125}I]-endothelin in the absence and presence of 100 nM unlabeled endothelin to equilibrium. Data points represent means of duplicate determinations. The means for receptor density (R_O) and equilibrium dissociation constant (K_d) derived from three separate experiments are shown.

108 ± 13 pM in glomerular and papillary membranes, respectively (n = 3). Endothelin 3 and sarafotoxin S6b were nearly equipotent with endothelin 1 in competing for glomerular endothelin binding sites (Figure 3). Deletion of the terminal tryptophan of endothelin 1 lowered the affinity for the binding sites 100-fold. Endothelin-like peptide, which has none of the last 6 carboxy-

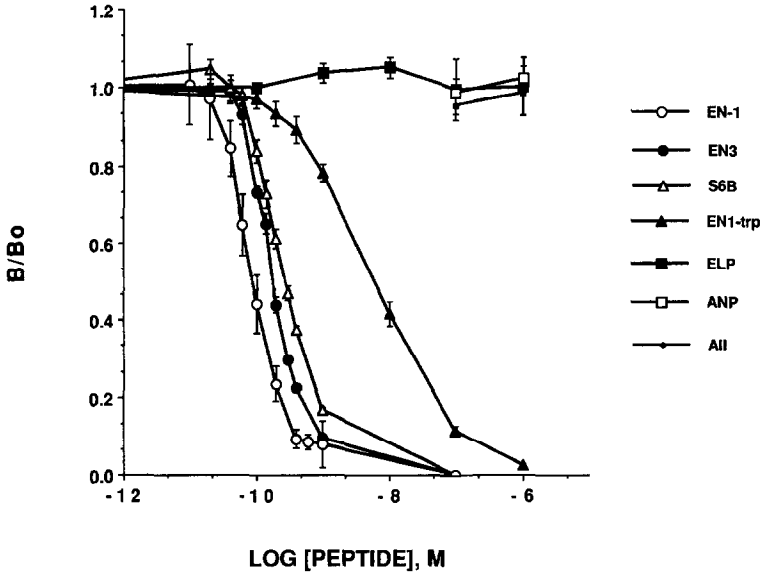
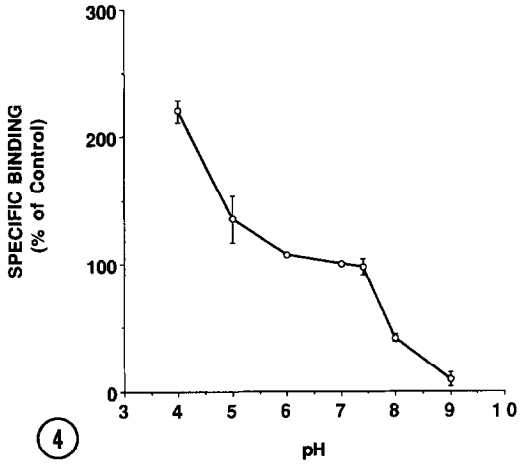


Figure 3. Competitive inhibition of [^{125}I]-endothelin binding by unlabeled endothelin and various other peptides in glomerular membranes. Membranes were incubated with [^{125}I]-endothelin in the presence of increasing concentrations of unlabeled endothelin or other peptides. Inhibition is expressed as the ratio of radioactivity bound in presence of competitor to that bound in its absence (B/Bo). Half-maximal inhibition occurred at 140 ± 22 pM for endothelin 1 (EN-1). Other peptides shown are endothelin 3 (EN3), sarafotoxin S6b (S6B), a tryptophan-deleted endothelin 1 (EN1-trp), endothelin-like peptide (ELP), angiotensin II (AII), and atrial natriuretic peptide (ANP).



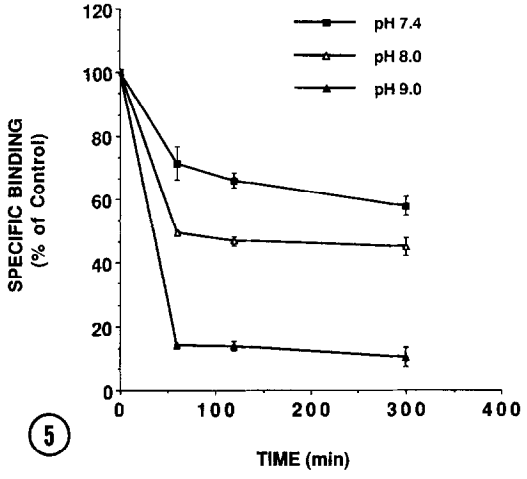


Figure 4. Effect of pH on endothelin binding. A constant concentration of glomerular membrane was incubated for 18 hours at 4°C at various pH from 4.0 to 9.0. Data points represent means of three separate experiments, each done in duplicate. Control (100 %) was arbitrarily assigned at pH 7.0.

Figure 5. Effect of pH on dissociation of endothelin binding in papillary membranes. A constant concentration of papillary membrane was incubated for 18 hours at 4°C at pH 7.4. At time 0, dissociation was initiated by changing the incubation temperature to 25°C, adding excess unlabeled endothelin (300 nM), and by adjusting the assay conditions as indicated. Data points represent means of three separate experiments, each done in triplicate. Control (100 %) was defined as binding at time 0.

terminus residues homologous to endothelin 1, failed to compete for binding even in the micromolar range. Atrial natriuretic peptide, angiotensin II, and platelet-derived growth factor did not compete for binding indicating specificity of the sites for endothelin.

The effect of Mg^{+2}, Ca^{+2}, Mn^{+2} and GTPγS on endothelin binding to papillary membranes was also examined. Mn^{+2} increased [^{125}I]-endothelin binding by 46.9 ± 0.1 % (n = 3; data not shown) whereas Ca^{+2} and Mg^{+2} were without effect. Moreover, addition of GTPγS to a final concentration of 1 mM failed to alter significantly specific [^{125}I]-endothelin binding to either glomerular or papillary membranes (data not shown).

Figure 4 shows the pH dependency of [^{125}I]-endothelin binding to glomerular membranes. Binding was maximal at pH 4 and was markedly inhibited as the pH was raised above 8.

We next examined the effect of pH on dissociation of endothelin binding in papillary membranes. A constant concentration of papillary membranes was incubated for 18 hours at 4°C at pH 7.4. At time 0, dissociation was initiated by changing the incubation temperature to 25°C, adding excess unlabeled endothelin and by adjusting the assay conditions as indicated (Figure 5). As is shown, endothelin binding was reversible especially at pH 9.0, but dissociation rates were extremely slow at pH 7.4 and intermediate at pH 8.0.

To assess how many subtypes of endothelin binding sites existed as well as the molecular mass of each, [^{125}I]-endothelin preincubated with isolated glomeruli was covalently linked to its binding sites with the bifunctional reagent *Bis* (sulfosuccinimidyl) suberate. After solubilization and SDS-PAGE, autoradiography of the 7.5 % polyacrylamide gel revealed a predominant labeled

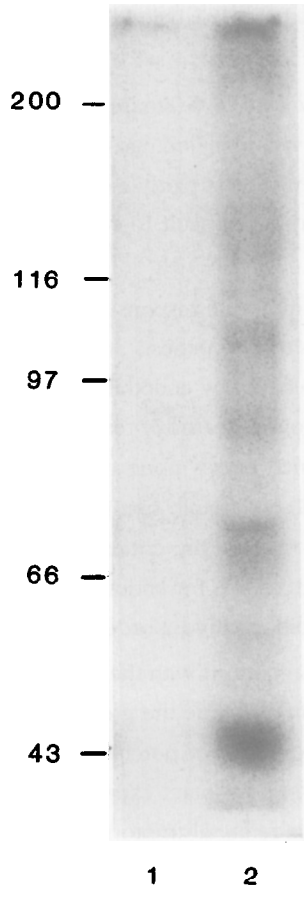


Figure 6. Autoradiogram of sodium dodecyl sulfate-polyacrylamide gel electrophoresis of isolated glomeruli incubated with and subsequently covalently linked to [^{125}I]-endothelin in the presence (lane 1) and in the absence (lane 2) of 100 nM unlabeled endothelin. The predominant specifically labeled band is at 45 kDa. Bands were also observed at 69, 86, 107, and 128 kDa. The same bands were observed when either glomerular or papillary membranes were used in the presence or absence of reducing conditions.

band of estimated molecular masses of 45 kDa (Figure 6). Faintly labeled bands were also observed at 69, 86, 107 and 128 kDa (Figure 6). Labeling was absent when 100 nM endothelin was added to the preincubation.

DISCUSSION

Because of the reported effects of endothelin on rat glomerular and papillary collecting duct function, the present study was undertaken to identify and characterize specific endothelin binding sites in membranes of these target tissues.

We found these binding sites to be saturable and of high affinity. The dissociation constants, which averaged 662 ± 151 and 1309 ± 123 pM for papillary and glomerular membranes, respectively, are within the same order of magnitude as those found by other investigators in vascular smooth muscle and adrenal zona glomerulosa cells (6, 12-15). These dissociation constants, however, are 5 to 10-fold greater than the IC_{50} derived from competition binding

inhibition studies, suggesting that the radiolabeled ligand has a lower affinity for the binding site than does the native ligand.

Binding is reversible especially at pH 9.0 (Figure 5). Although dissociation also occurs at pH 7.4, it is much slower, consistent with the findings for vascular smooth muscle cells (12). This remarkable stability in binding may at least partly explain the experimental observations that the contractile response to endothelin is difficult to reverse with a perfusate wash (6) and is of prolonged duration in vivo (9).

The domain of the endothelin molecule responsible for its specific binding appears to be the carboxy-terminus. Consistent with this hypothesis are the curves describing the relative order of potency of competitive binding inhibition by endothelin 1 and analogues (Figure 3). Endothelin 3 and endothelin 1 share a homologous carboxy-terminus and have similar binding affinities. Endothelin-like peptide (ELP), which shares none of the last 6 carboxy-terminus amino acids, failed to competitively inhibit [^{125}I]-endothelin binding. Sarafotoxin S6b, which shares 4 of the last 6 carboxy-terminus residues including the critical terminal dipeptide isoleucine-tryptophan, binds with an affinity almost as high as that for endothelin 3. Deletion of the terminal tryptophan shifts the competitive binding inhibition curve 2 orders of magnitude to the right.

Published physiolgical studies, in concert with the above observations, suggest that the higher the binding affinity of an endothelin analogue the greater its potency as an agonist. Kimura et al (18) investigated the constrictive response of porcine coronary strips to endothelin 1 and some of its derivatives and discovered that an intact carboxy-terminus was required for activity. In these vascular strips, deletion of the terminal tryptophan also shifted the dose-response curve 3 orders of magnitude to the right. Furthermore, additional deletion of the adjacent isoleucine shifted the curve even further to the right. Without the last 6 carboxy-terminus residues, no physiological activity was observed.

The binding affinity of many hormone receptors which are associated with G proteins is reduced in the presence of nonhydrolyzable GTP analogues (20). Because specific endothelin binding failed to be modulated by GTPγS, it is unlikely that these binding sites are coupled to a G protein. Furthermore, hormone receptor binding is generally inhibited at an acidic pH and cellular processing of many internalized hormone-receptor complexes involves their dissociation in acidic vesicles allowing some receptors to recycle to the cell surface (19). The curve describing the pH dependence of endothelin binding to glomerular membranes (Figure 4) is a mirror image of what would be predicted. This peculiar pH optimum, not characteristic of cell-surface receptors, is more consistent with intracellular binding sites.

Although the results of our saturation studies suggest sites of uniform affinity, Scatchard analysis cannot distinguish between single and multiple receptor subtypes with similar affinities. When the binding sites are covalently linked to [^{125}I]-endothelin and then subjected to SDS-PAGE, multiple specifically labeled bands are observed. The predominant band of molecular mass of 45 kDa is unlikely to be a transmembrane protein. These multiple bands most likely represent distinct endothelin binding proteins of which only one, or perhaps two, of highest molecular mass is the physiologic cell-surface receptor. In support of this explanation are results from fractionation of papillary membranes by centrifugation on a sucrose density gradient.

Specific ANP binding is highly enriched in the lightest fraction where plasma membrane is also found. In contrast, specific endothelin binding is not localized to any specific fraction (data not shown). Elucidation of the exact nature of the endothelin receptor will require binding and covalent linking of the radioligand to intact cells under conditions which minimize internalization.

ACKNOWLEDGMENTS: This work was supported by grants 1R29-DK40445 and R01-DK35930 from the National Institutes of Health. E.R. Martin is a recipient of an NIH Individual National Research Service Award # 1-F32-DK08196-01. P.A. Marsden is a Medical Research Council of Canada fellowship recipient.

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