Interaction of Endothelin-1 with Cloned Bovine ETA Receptors: Biochemical Parameters and Functional Consequences†

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ABSTRACT: This paper defines the properties of interaction of endothelin-1 (Et-1) with cloned bovine ETA receptors. The K_d value of Et-1/ETA receptor complexes was estimated in membrane preparations to 20 pM using kinetic experiments and saturation experiments performed under quasi equilibrium conditions. Competition experiments yield a wide range of apparent K_d(Et-1) values from 20 pM to 1 nM which were in fact measures of the receptor concentrations rather than of K_d values. This resulted from the fact that complex second-order rate kinetics rather than pseudo-first-order kinetics control the association of Et-1 to its receptor when the receptor concentration is larger than K_d(Et-1). Et-1 induced a production of inositol phosphates with an apparent affinity of 2.3 nM, 100 times higher than the K_d-(Et-1) value determined previously. Numerical simulation suggested that under time-limited conditions, sub-nanomolar rather than picomolar concentrations of Et-1 are necessary to occupy an important fraction of picomolar sites. It is concluded that bovine ETA receptors have a single affinity state for Et-1 (K_d = 20 pM) and that this affinity state can account for nanomolar actions of Et-1 in intact cells. It is suggested that the sensitivity of a preparation to Et-1 is a cell property rather than a receptor property. It is also suggested that the main advantage of high-affinity Et-1 binding is to promote autocrine actions rather than a high potency of the peptide.

Endothelins (Et)[1] are a family of 21 amino acid peptides that are produced in a variety of tissues and act as modulators of the vascular tone, cell proliferation, and hormone production (Yanagisawa et al., 1988; Rubanyi & Polokoff, 1994). They recognize two forms of receptors, namely, the ETA and ETB receptor subtypes (Arai et al., 1990; Sakurai et al., 1990), that are seven-transmembrane domain receptors that couple to different intracellular signaling pathways via heterotrimeric G proteins. Endothelin receptors are coupled to phospholipases C and A2 and adenylyl cyclase. Numerous studies performed using endothelins and specific receptor antagonists have suggested that these peptides are important in vascular physiology and diseases (Rubanyi & Polokoff, 1994). Their mechanisms of action are however complex, and many aspects of the physiology of endothelins are still poorly understood. Among numerous examples is the observation that in heterozygous Et-1 +/− mice, decreased circulating Et-1 levels are associated with a mild increase in blood pressure rather than with the decrease expected from its vasoconstrictor action (Kurihara et al., 1994).

Et-1 binds with a high affinity to its receptors, and it is usually believed that high-affinity binding determines high-affinity responses. In spite of numerous studies, the K_d value of Et-1 for its receptor is not known with certainty. Values as low as a few picomolars and as high as a few nanomolars have been reported. This variability has been observed with both endogenous receptors and transfected receptors. Actions of Et-1 are also observed in a wide range of concentrations. In some preparations, picomolar concentrations of Et-1 are active (Serradeil-Le Gal et al., 1991; Shirakami et al., 1993; Shraga-Levine et al., 1994; Sokolovsky et al., 1994; Journeaux et al., 1994). In many preparations, nanomolar concentrations of Et-1 are necessary to elicit an action. This variability had led to the suggestion that several subtypes of Et receptors (with superhigh, high, and low affinities for Et-1) mediate different actions of the peptide. The difficulty with this hypothesis is that molecular cloning experiments have identified only two forms of receptors: the Et-1 selective ETA receptor and the Et isopeptide nonselective ETB receptor (Sakamoto et al., 1991).

The present study was performed to define the properties of interaction of Et-1 with ETA receptors, to look for possible receptor heterogeneities, and to analyze the functional consequences of high-affinity Et-1 binding. It was performed using fibroblasts transfected with cloned bovine ETA receptors.

EXPERIMENTAL PROCEDURES

Materials. Et-1 and BQ-123 were purchased from Neosystems (Strasbourg, France). [^{3}H]BQ-123 (42.7 Ci/mmol) was synthesized at Roussel Uclaf. ^{125}INa (2200 Ci/mmol) was from NEN. *myo*-[2-^{3}H]Inositol (19 Ci/mmol) was from Amersham. All reagents were from the Sigma Chemical Co. unless otherwise indicated.

Iodination. [^{125}I]Et-1 (2200 Ci/mmol) was obtained from Amersham Corp. or prepared using chloramine T. Briefly, 4 nmol of Et-1 dissolved into 0.05 M Na phosphate/boric acid buffer at pH 7.4 was mixed with 0.7 nmol (1.5 mCi) of ^{125}INa and 20 nmol of freshly prepared chloramine T. After

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[1] Abbreviations: Et, endothelin.

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30 s, 500 nmol of tyrosine was added; the reaction mixture (280 μL) was diluted with 700 μL of a solution consisting of 10% acetonitrile, 0.05% triethanolamine, and 0.1% trifluoroacetic acid and injected on a C18 Lichrocart 250-4 column (Merck). The column was eluted with a linear gradient of 10−50% acetonitrile containing 0.05% triethanolamine and 0.1% trifluoroacetic acid for 60 min at a rate of 1 mL/min. Optical density at 275 nm and radioactivity were followed on line, and 1 mL fractions were collected into low-absorption tubes containing 100 μL of a 1 M Tris solution (pH 8.0) supplemented with 0.2% bovine serum albumin.

Cell Cultures. A bovine ETA receptor cDNA clone was kindly provided by Dr. S. Nakanishi. Stable transfectant CCl39 fibroblasts were kindly provided by Dr. J. Pouyssegur. Cells were grown into Dulbecco's modified Eagle's medium supplemented with 7.5% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin.

Cell Homogenates. All operations were performed at 4 °C. Cells prepared using 100 mm culture dishes were washed and scrapped with a rubber policeman into the homogenization buffer (250 mM sucrose, 1 mM EDTA, 20 mM Hepes/NaOH at pH 7.4) and supplemented with a cocktail of protease inhibitors (0.1 mM bacitracin, 0.1 mM phenylmethanesulfonyl fluoride, 1 μM leupeptine). The extract was homogenized three times for 5 s using a Polytron (position 6) and centrifuged at 125000*g* for 45 min. The pellet was collected, diluted into the homogenization buffer, and stored at −20 °C. Cell homogenates were used in kinetic and competition experiments. Purified membranes were used in some experiments (e.g., Figure 3). They were prepared by first centrifuging cell homogenates at 1000*g* (10 min). The supernatant was then recovered, and membranes were pelleted by centrifigation at 20000*g* (30 min). Proteins were determined according to Bradford (Bradford, 1976).

Binding Experiments on Cell Membranes. All experiments were performed at 20 °C. Cell homogenates (0.5−166 μg of protein/mL) were diluted into an Earle's salt solution (140 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 25 mM Hepes/Tris at pH 7.4) supplemented with the cocktail of protease inhibitors and the desired concentrations of [^{125}I]-Et-1 or of unlabeled Et-1. After selected times of incubation, aliquots of the incubation solution were filtered under reduced pressure onto Sartorius (Palaiseau, France) 0.2 μm filters and washed three times with 4 mL of 0.1 M $MgCl_2$. Filters were then counted. Nonspecific binding was determined in parallel experiments using 100 nM unlabeled Et-1.

For [^{3}H]BQ-123 binding experiments, cell homogenates (0.2 mg of protein/mL) were incubated in the presence of varying concentrations of [^{3}H]BQ-123 for 1 h at 20 °C and the bound radioactivity was measured as described above. Nonspecific binding was defined in the presence of 10 μM unlabeled BQ-123.

Binding to Intact Cells. All experiments were performed at 37 °C. Cells were seeded into 24-well tissue culture clusters and used at confluency unless otherwise indicated. Washed cells were incubated into an Earle's salt solution containing [^{125}I]Et-1. Incubations were stopped by washing cells six times with 0.2 mL of 0.1 M $MgCl_2$. Cells were then collected into 0.1 N NaOH, and the cell-associated radioactivity was counted. Nonspecific binding was determined in parallel experiments using 100 nM unlabeled Et-1. We checked that >95% of the extracellular radioactivity remaining at the end of the incubation was unmodified [^{125}I]-Et-1.

Internalization of Et-1/receptor complexes was documented by the acid wash technique (Resink et al., 1990). After different times of association with [^{125}I]Et-1, cells were exposed twice for 10 min to an ice cold solution consisting of 0.5 M NaCl acidified to pH 2.5 with acetic acid. Cells were then digested into 0.1 N NaOH and treated as above.

Phospholipase C Activity. Cells (40 000/well) were seeded into 96-well tissue culture clusters in DMEM supplemented with 7.5% fetal bovine serum and allowed to grow for 24 h. Culture media were then changed to a serum free culture medium supplemented with 2 μCi/mL [^{3}H]inositol. After 24 h, cells were rinsed and further incubated for 15 min into Hepes-buffered DMEM supplemented with 20 mM LiCl, 0.2% lysozyme, and the desired concentrations of Et-1. Cells were then extracted with 10 mM formic acid, and inositol phosphates were separated on Dowex AG1 × 8 anion exchange columns (Biorad). Columns were sequentially eluted with 3 mM NH_4OH, 40 mM ammonium formate, and 2 M ammonium formate. The last fraction, corresponding to total inositol phosphates, was counted.

Data Analysis. Data were analyzed by nonlinear regression using Sigma Plot (Jandel Corp.).

Numerical Similation of Et-1 Binding. We considered that receptors (R) were mixed with a labeled ligand (L*) and the corresponding nonlabeled ligand (L). Rates of formation of RL and RL* complexes, given by

$$\frac{d[RL]}{dt} = k_1[L][R] - k_{-1}[RL]$$

$$\frac{d[RL^*]}{dt} = k_1^*[L^*][R] - k_{-1}^*[RL^*]$$

were computed for 5 s intervals. RL and RL* concentrations were cumulated over a 15 min period using the Excel software (Microsoft Corp.). The time chosen corresponded to the time at which the production of inositol phosphates was measured. Parameters used were k_1([^{125}I]Et-1) = 2.7 × 10^{-4} pM^{-1} min^{-1}, k_{-1}(Et-1) = k_{-1}([^{125}I]Et-1) = 5 × 10^{-3} min^{-1}, [R_o] = 300 pM, and [[^{125}I]Et-1] = 20 pM. Different k_1(Et-1) values were considered.

RESULTS

Association Experiments. Consider a simple bimolecular reaction scheme in which a receptor (R) and its labeled ligand (L*) are mixed and allowed to form reversible complexes (RL*). [R_o] and [L^*_o] are the initial concentrations of R and L*, respectively. The equilibrium dissociation constant (K_d) of RL* complexes is the ratio of the first-order rate constant of dissociation (k_{-1}) to the second-order rate constant of association (k_1). It is usual to define these parameters from a pseudo-first-order plot of association data and a first-order plot of dissociation data. This procedure was not satisfactory for two reasons: (i) Dissociation kinetics are very slow, and accurate estimates of k_{-1} values could not be obtained from dissociation data, and (ii) at both low R_o and L^*_o concentrations (two conditions that are necessary to apply pseudo-first-order approximations), binding equilibrium develops very slowly and equilibrium [RL*] could

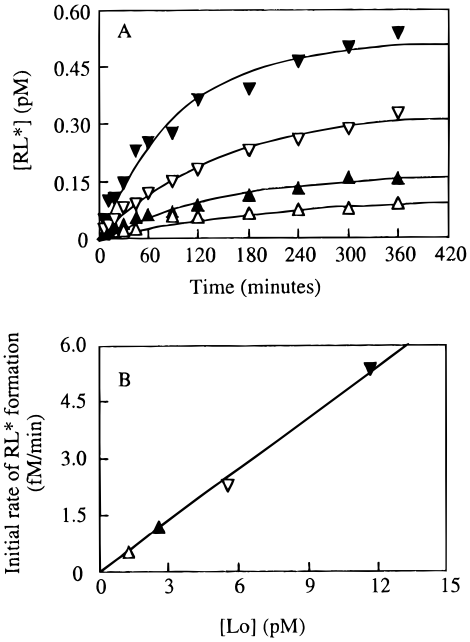

FIGURE 1: Kinetics of [^{125}I]Et-1 binding. Panel A: Cell homogenates (1 μg of protein/mL) were incubated with 1.3 pM (△), 2.6 pM (▲), 5.5 pM (▽), or 11.6 pM (▼) [^{125}I]Et-1, and the specific binding component was determined after different times of incubation. Each series of experimental points was fitted by nonlinear regression to eq 1 to yield parameters *a* and *b*. Panel B: Relationship beween the initial rate of RL* formation (a) and [L*$_o$]. The slope of the relationship (4.5×10^{-4} min^{-1}) is a measure of k_1[R$_o$]. Symbols have the same meaning as in panel A.

not be defined with precision. For these reasons, we analyzed initial rates of [^{125}I]Et-1 binding.

Under conditions in which [RL*] $\ll$ [R$_o$] + [L*$_o$], the formation of RL* complexes follows an exponential course of the form:

$$[RL^*] = a(1 - \exp(-bt)) \quad (1)$$

$$\text{where } a = \frac{[R_o][L^*_o]}{K_d + [R_o] + [L^*_o]} \quad (2)$$

$$\text{and } b = k_{-1} + k_1([R_o] + [L^*_o]) \quad (3)$$

Figure 1A shows the results of experiments in which cell homogenates were incubated for different periods of time in the presence of different concentrations of [^{125}I]Et-1. The time course of [^{125}I]Et-1 binding followed an exponential course in all conditions tested. Analysis of the data by nonlinear regression yields parameters *a* and *b* for each curve. As expected, the initial rate of RL* formation (d[RL*]/dt = k_1[R$_o$][L*$_o$]) was linearly related to [L*$_o$] (Figure 1B). The slope of the relationship (4.5×10^{-4} min^{-1}) was a measure of k_1[R$_o$].

Figure 2 further shows that, as expected from eq 3, parameter *b* was linearly related to [L*$_o$]. The slope of the representation (k_1) was 2.7×10^{-4} pM^{-1} min^{-1}. The ordinate at the origin (5×10^{-3} min^{-1}) was a measure of k_{-1} + k_1[R$_o$]. From the k_1[R$_o$] value defined previously, k_{-1} was estimated to 4.5×10^{-3} min^{-1}. This value corresponded to a half-life of RL* complexes (ln 2/k_{-1}) of 2.5 h. This procedure also allowed us to estimate [R$_o$]. This value was 1.8 pM, corresponding to 1.8 pmol/mg of protein.

The equilibrium dissociation constant of [^{125}I]Et-1/ETA receptor complex (K_d([^{125}I]Et-1) = k_{-1}/k_1) was therefore

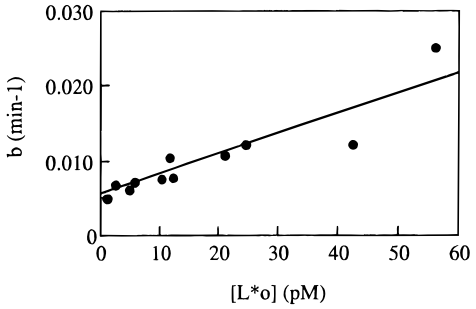

FIGURE 2: Relationship between the exponential constant *b* and [L$_o$]. Parameters *b* derived from 11 kinetic experiments such as those shown in Figure 1 are plotted against [[^{125}I]Et-1]. The slope of the representation is a measure of k_1 (2.7×10^{-4} pM^{-1} min^{-1}). The ordinate at the origin (5×10^{-3} min^{-1}) is a measure of k_{-1} + k_1[R$_o$].

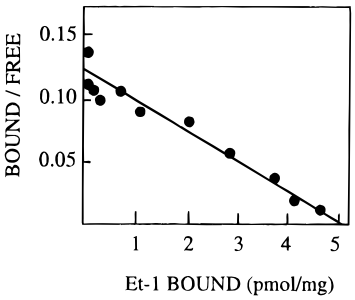

FIGURE 3: Scatchard plot for the specific [^{125}I]Et-1 binding to membranes. Membranes (0.5 μg of protein/mL) were incubated in the presence of varying concentrations of [^{125}I]Et-1 for 3 h, and the bound radioactivity was measured. Nonspecific binding was defined in the presence of 100 nM unlabeled Et-1. The free [^{125}I]-Et-1 concentration was calculated by substracting bound [^{125}I]Et-1 from the total [^{125}I]Et-1 concentration. From this representation, K_d was estimated to be 16 pM. Maximum binding capacity was 4.9 pmol/mg of protein corresponding to [R$_o$] = 2.45 pM. Similar experiments performed with cell homogenates yield a lower maximum binding capacity (1.0−1.4 pmol/mg of protein). Points are means of triplicates.

estimated to 17 pM. This calculation assumes a one-step binding reaction with no isomerization.

Scatchard Analysis. K_d([^{125}I]Et-1) can be derived from a Scatchard analysis of saturation experiments performed under equilibrium binding conditions. Knowing that the half-life of [^{125}I]Et-1/receptor complexes was 2.5 h (see above), an incubation time > 20 h would be necessary to reach binding equilibrium. Long incubation times were associated to a partial degradation of [^{125}I]Et-1 that could not be prevented by protease inhibitors. We therefore reduced times of equilibrations to 3 h. Under these conditions, less than 5% of initially added [^{125}I]Et-1 was degraded.

Figure 3 shows a linear Scatchard plot that indicated the presence of a single family of noninteracting [^{125}I]Et-1 binding sites. The slope of the relationship indicated a K_d-([^{125}I]Et-1) value of 16 pM, in good agreement with the K_d-([^{125}I]Et-1) value obtained in kinetic experiments (17 pM).

Competition Experiments. Competition experiments in which membranes are incubated in the presence of [^{125}I]Et-1 and different concentrations of unlabeled Et-1 are useful to define the equilibrium dissociation constant of unlabeled Et-1/receptor complexes. Figure 4A shows that Et-1 dose dependently prevented [^{125}I]Et-1 binding in monophasic manners. No evidence of biphasic competition curves could be obtained. It also shows that concentrations of Et-1 necessary to inhibit 50% of the specific [^{125}I]Et-1 binding

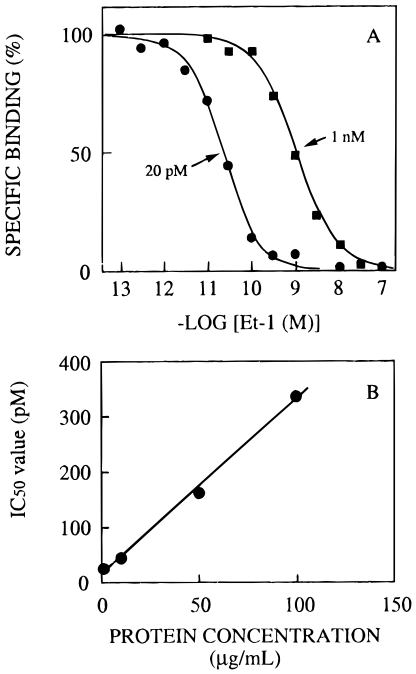

FIGURE 4: Competition experiments using membrane preparations. Panel A: Typical dose−response curves for Et-1 inhibition of the specific [^{125}I]Et-1 binding to cell homogenates. Membranes (at 1 μg/mL (●) or 166 μg/mL (■)) were incubated for 3 h in the presence of 5.7 pM [^{125}I]Et-1 and the indicated concentrations of unlabeled Et-1, and the specific binding component was determined. Points are means of triplicates. Panel B: Relationship between IC_{50} values for Et-1 inhibition of the specific [^{125}I]Et-1 binding component and the protein concentration used in the assay. These experiments were performed using the same membrane preparation and 20 pM [^{125}I]Et-1. The extrapolated IC_{50} value at infinite dilution of the membranes was 20 pM. Means of two independent experiments are shown.

(IC_{50}) as high as 1 nM and as low as 20 pM were obtained in experiments performed at different membrane dilutions, i.e., at different [R_o]. A dependence of IC_{50} values on [R_o] is not usual. It has however been recognized long ago that a high [R_o] condition is a major source of artifacts for characterizing high-affinity binding (Jacobs et al., 1975). The general formula relating the apparent dissociation constant of a competitor ligand for its receptor (K'_d, defined as the concentration of free unlabeled ligand at which 50% of the specific binding component is inhibited) to its true dissociation constant (K_d) is

$$K'_d = K_d + \frac{K_d}{K_d^*}([L^*_o] + [R_o] - {}^3/_2[RL^*]) \qquad (4)$$

where K_d* is the equilibrium dissociation constant of RL* complexes, [RL*] is the concentration of bound labeled ligand in the absence of unlabeled ligand, [L^*_o] is the initial concentration of labeled ligand, and [R_o] is the initial receptor concentration (Jacobs et al., 1975). When [R_o] $\ll$ K_d, eq 4 simplifies and gives the well-known Cheng−Prussoff relationship (Cheng and Prusoff, 1973):

$$K'_d = K_d\left(1 + \frac{[L^*_o]}{K_d^*}\right) \qquad (5)$$

When [R_o] > K_d, a nonnegligible fraction of the initial ligand concentration binds to receptors and the free ligand concentration is overestimated by a factor which is dependent on the bound radioactivity, i.e., on [R_o]. When [R_o] is larger than K_d by a factor of at least 100, all ligand molecules are trapped onto receptors. This condition is known as a stoichiometric (or tight binding) condition (Goldstein, 1944; Henderson, 1972).

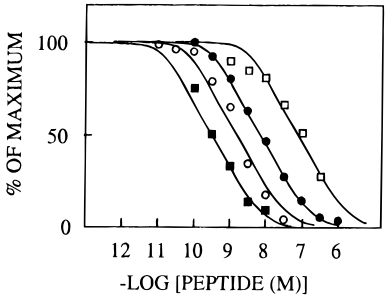

FIGURE 5: Inhibition by unlabeled Et isopeptides of the specific [^{125}I]Et-1 binding. Cell homogenates (10 μg of protein/mL) were incubated for 3 h in the presence of 8 pM [^{125}I]Et-1 and the indicated concentrations of unlabeled peptides. The specific [^{125}I]Et-1 binding component was then determined. Symbols used are sarafotoxin S6b (■), BQ-123 (○), Et-3 (●), and sarafotoxin S6c (□). Points are means of triplicates.

Figure 4B presents the results of experiments in which competition curves were established for the same membrane preparation and using identical concentrations of [^{125}I]Et-1. It shows that measured IC_{50} values were linearly related to the amount of protein used in the assay. At infinite dilution of the membranes, IC_{50} values extrapolated down to 20 pM (Figure 4B). This value was close to the K_d([^{125}I]Et-1) values determined in kinetic and saturation experiments. Thus picomolar values of K_d(Et-1) could be obtained in competition experiments, but this required the use of very diluted membrane preparations. At high [R_o] values, IC_{50} values are not measures of K_d. They are measures of [R_o].

The pharmacological profile of Et-1 binding sites was defined in competition experiments. Figure 5 shows that unlabeled Et isopeptides prevented [^{125}I]Et-1 binding with the following rank order of potency: sarafotoxin S6b (IC_{50} = 300 ± 36 pM) > BQ-123 (IC_{50} = 2 ± 0.5 nM) > Et-3 (IC_{50} = 8 ± 2 nM) > sarafotoxin S6c (IC_{50} = 100 ± 24 nM). True affinities were determined using eq 4. They were 150 pM and 1, 4, and 50 nM for sarafotoxin S6b, BQ-123, Et-3, and sarafotoxin S6c, respectively. This profile is characteristic of an ETA receptor subtype.

Binding Properties of [^{3}H]BQ-123. Figure 6 shows a Scatchard plot for the specific [^{3}H]BQ-123 binding to cell homogenates. It indicated the presence of a single family of binding sites with a K_d value of 1.25 nM, in good agreement with the K_d value for BQ-123 determined in competition assays against [^{125}I]Et-1 (1 nM, Figure 5). The maximum [^{3}H]BQ-123 binding capacity was 1.3 pmol/mg of protein, close to the maximum [^{125}I]Et-1 binding capacity obtained in the same preparation of cell homogenate (1.4 pmol/mg of protein).

Use of an Intermediate Ligand. An important point to consider when dealing with high-affinity binding is the possible existence of stoichiometric (or tight binding) conditions. Under such conditions, unusual binding isotherms arise and all approaches that are classically used to define K_d values are misleading (Goldstein, 1944; Henderson, 1972). One method for defining a K_d value under very general conditions, which include stoichiometric binding, is to mix similar concentrations of R and L* in the presence of a large

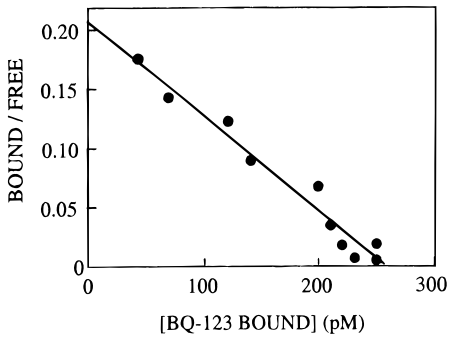

FIGURE 6: Scatchard plot for the specific [^{3}H]BQ-123 binding to membranes. Cell homogenates (0.2 mg of protein/mL) were incubated in the presence of varying concentrations of [^{3}H]BQ-123 for 1 h, and the bound radioactivity was measured. Nonspecific binding was defined in the presence of 1 μM unlabeled BQ-123. From this representation, K_d was estimated to 1.25 nM. Maximum binding capacity was 1.3 pmol/mg of protein. Points are means of triplicates.

excess of competitor drug whose binding is reversible and well defined. This approach has been used successfully to define the interaction of trypsin with the pancreatic trypsin inhibitor. The complexes formed dissociate with a half-life of the order of 4.5 months (Vincent and Lazdunski, 1972). BQ-123 was suitable for this type of experiment for it binds rapidly and reversibly to ETA receptors. Parameters describing the interaction of BQ-123 with ETA receptors have been defined previously (Ihara et al., 1995). They are $k_1 = 0.34$ nM^{-1} min^{-1} and $k_{-1} = 0.5$ min^{-1}. These correspond to a half-life of BQ-123/receptor complexes of 1.4 min and to a K_d value of 1.5 nM, close to the value determined from equilibrium binding experiments (1 nM, Figures 5 and 6).

ETA receptors (1.35 nM, defined from [^{3}H]BQ-123 binding experiments) were mixed for 30 min with 100 nM unlabeled BQ-123. It was calculated that these conditions were sufficient to reach equilibrium binding and saturate receptors. [^{125}I]Et-1 (1.16 nM) was then added to the reaction mixture, and its binding was followed. After 2 h of incubation at 20 °C, the concentration of [^{125}I]Et-1/receptor complexes reached a maximum which was estimated to 270 pM. The K_d value for BQ-123/receptor complexes is related to the equilibrium concentrations of R (which is very low and cannot be assessed), BQ-123 (100 nM), and receptor/BQ123 complexes ([R$_o$] − [R/[^{125}I]Et-1], i.e., 1.08 nM) by the relationship:

$$K_d(\text{BQ-123}) = \frac{[\text{R}][\text{BQ-123}]}{[\text{R/BQ-123}]} \quad (6)$$

Conversely, the K_d value for the [^{125}I]Et-1/receptor complexes is related to the equilibrium concentrations of R, [^{125}I]Et-1 (890 pM), and R/[^{125}I]Et-1 complexes (270 pM):

$$K_d([^{125}\text{I}]\text{Et-1}) = \frac{[\text{R}][[^{125}\text{I}]\text{Et-1}]}{[\text{R}/[^{125}\text{I}]\text{Et-1}]} \quad (7)$$

Solving the two equations yields

$$\frac{K_d(\text{BQ-123})}{K_d([^{125}\text{I}]\text{Et-1})} = \frac{[\text{BQ-123}][\text{R}/[^{125}\text{I}]\text{Et-1}]}{[[^{125}\text{I}]\text{Et-1}][\text{R/BQ-123}]} \quad (8)$$

Data obtained indicated a K_d(BQ-123)/K_d([^{125}I]Et-1) ratio of 28. The K_d value of BQ-123/receptor complexes being 1 nM (Figures 5 and 6), it follows that K_d([^{125}I]Et-1) = 36 pM. This value is close to K_d values determined from kinetic and quasi equilibrium binding studies. It indicated that nanomolar concentrations of [^{125}I]Et-1 detected picomolar sites and not nanomolar sites.

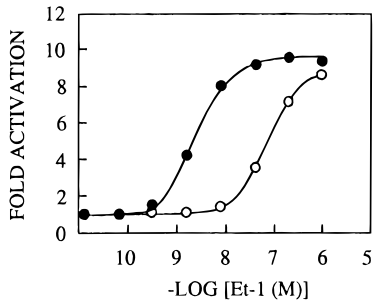

FIGURE 7: Dose−response curve for Et-1-induced activation of phospholipase C and its inhibition by BQ-123. Cells were exposed to the indicated concentrations of Et-1 in the absence (●) and presence (○) of 5 μM BQ-123. The production of total inositol phosphates was measured after 15 min. Means of four determinations are indicated. SE was smaller than the size of the symbols.

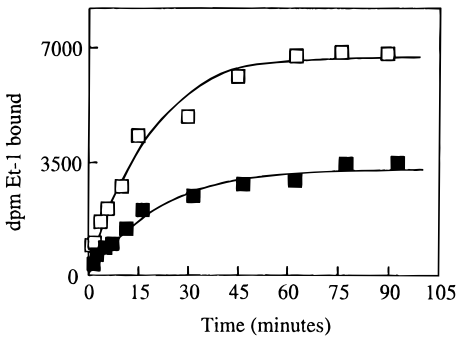

FIGURE 8: Internalization of [^{125}I]Et-1 by intact cells. Cells were incubated in the presence of 24 pM [^{125}I]Et-1. At different times of an association kinetics, cells were either washed with saline (□) or with acid (■) to dissociate membrane-bound label. The nonspecific binding component was substracted from the data. Points are means of triplicates.

Activation of Phospholipase C. Figure 7 shows that Et-1 induced a production of inositol phosphates from prelabeled inositol phospholipids in a dose dependent manner. In six independent experiments using confluent cell monolayers, the concentration for half-maximum activation of phospholipase C (EC_{50}) was 2.3 ± 0.4 nM. Figure 7 also shows that the dose−response curve for Et-1 action was shifted to higher concentrations in the presence of BQ-123. This result was expected for an ETA receptor subtype.

Properties of Interaction of [^{125}I]Et-1 with Intact Cells. The previous results indicated a 100-fold difference between the EC_{50} value for Et-1-induced activation of phospholipase C (2.3 nM) and the K_d value of Et-1/receptor complexes (20 pM) determined in membrane preparations. The difference may suggest that intact cells provided an environment that was different from that of membrane preparations and that modified the affinity of ETA receptors for Et-1.

A possible source of intricacy is internalization of Et-1/receptor complexes (Resink et al., 1990; Marsault et al., 1993). Figure 8 shows that 50% of the bound [^{125}I]Et-1 could not be dissociated by an acidic treatment at any time of an association kinetic to intact cells. Knowing that in membrane preparations the same acid treatment dissociated >95% of preformed complexes within 2 min (data not shown), an obvious conclusion was that transfected fibro-

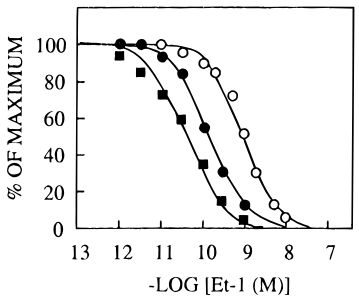

FIGURE 9: Competition experiments using intact cells. Representative dose−response curves for Et-1 inhibition of the specific [^{125}I]-Et-1 binding to intact cells. Experiments were performed using pure cultures of transfected fibroblasts seeded at two densities (corresponding to $[R_o] = 4$ pM (●) and 300 pM (○)) and with confluent monolayers of mixed transfected cells and untransfected parent cells (■, at 1/500 ratio corresponding to $[R_o] = 2$ pM). [[^{125}I]Et-1] was 10 pM (■), 20 pM (○), or 50 pM [^{125}I]Et-1 (●). The specific binding component was determined after 45 min. Means of triplicates are shown.

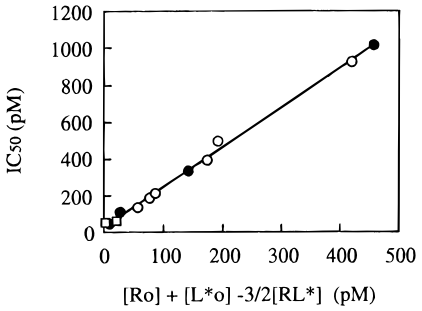

FIGURE 10: Relationship beween IC_{50} values obtained in competition experiments and $[R_o] + [L^*_o] - {}^3/_2[RL^*]$. IC_{50} values were obtained in competition experiments using membrane preparations (●), pure cultures of transfected cells at different dilutions (○), or cocultures of transfected and parent cells (□). They were plotted against $[R_o] + [L^*_o] - {}^3/_2[RL^*]$. The range of $[L^*_o]$ values covered in these experiments was 5−50 pM. The range of $[R_o]$ values covered was 0.5−450 pM. Some points in the low-abcissa range have been omitted for clarity. The ${}^3/_2[RL^*]$ parameter contributed little to the observed relationship.

blasts rapidly sequestred [^{125}I]Et-1/receptor complexes, presumably by internalization. It should be noted that sequestred sites are unable to release measurable amounts of [^{125}I]Et-1. As a consequence, no k_{-1} value can be defined for sequestred ligand/receptor complexes, and K_d values have no real meaning when intact cells are considered.

Figure 9 presents the results of competition experiments performed with confluent cell monolayers. The competition curve was monophasic. The IC_{50} value for Et-1 inhibition of the specific [^{125}I]Et-1 binding was 1 nM. This value is much larger than the K_d value of [^{125}I]Et-1/receptor complexes determined in membrane preparations. The possibility that the difference was due to a GTP effect was ruled out by the observation that GTPγS (0.1 mM) did not affect [^{125}I]-Et-1 binding to membranes. Another possibility is that, as discussed previously, the IC_{50} value was dependent on a high $[R_o]$ condition. $[R_o]$ was reduced either by seeding cells at a very low cell density or by using confluent monolayers of transfected fibroblasts that have been mixed with untransfected fibroblasts. Figure 9 shows that competition experiments performed with diluted cell preparations yielded IC_{50} values in the picomolar range rather than in the nanomolar range. The lowest IC_{50} value obtained at very low cell densities was 130 pM. The lowest IC_{50} value obtained with confluent cultures of transfected fibroblasts diluted with parent cells was 45 pM.

Equation 4 indicated that apparent affinities for competitor drugs are related to $[R_o] + [L^*_o] - {}^3/_2[RL^*]$. Figure 10 shows a plot of IC_{50} values as a function of $[R_o] + [L^*_o] - {}^3/_2[RL^*]$. The linearity of the plot indicated that different ($[R_o] + [L^*_o]$) conditions quantitatively accounted for the wide range of IC_{50} values observed both in membrane preparations and in intact cells. It also indicated that Et-1 recognized its receptors in intact cells and membrane preparations with similar properties.

The relationship extrapolated at the origin to a value of 50 pM. The slope of the relationship was markedly higher than 1. One reason is that IC_{50} values rather than K'_d values were used to plot the data. K'_d in eq 4 is defined as the concentration of free unlabeled ligand at which RL* is reduced by 50% (Jacobs et al., 1975). IC_{50} values used to plot the data relate to the initial rather than the free concentration of unlabeled ligand that reduces the formation of RL* by 50%. K'_d and IC_{50} values differ by a factor which

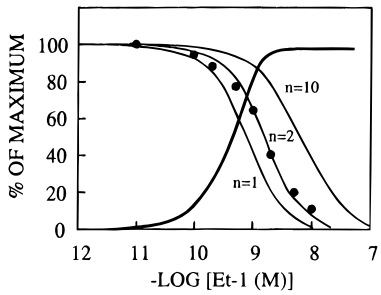

FIGURE 11: Modeling Et-1 binding and actions in intact cells. Symbols represent the results of a competition experiment in which cells (at a density corresponding to $[R_o] = 300$ pM) were exposed to 20 pM [^{125}I]Et-1 and the indicated concentrations of unlabeled Et-1. The specific binding component was determined after 15 min. This time corresponded to the time at which the production of inositol phosphates was measured and to the conditions used for the numerical simulation of Et-1 binding. The descending curves represent the fractional inhibition of [^{125}I]Et-1 binding calculated by numerical simulation using different values of k_1([^{125}I]Et-1)/k_1-(Et-1) ratio (n). The best fit of experimental data was obtained for a ratio of 2. Parameters used are defined in the Experimental Procedures. The ascending curve shows the expected fractional occupancy of the receptors by unlabeled Et-1 for a 15 min association. It was obtained using k_1(Et-1) $= 1.35 \times 10^{-4}$ pM^{-1} min^{-1} and k_{-1}(Et-1) $= 0$. Half-maximum site occupancy requires 0.5 nM Et-1.

is dependent on $[R_o]$ but which could not be corrected. Another reason for a slope > 1 could be that iodination of Et-1 slightly improved its affinity for ETA receptors (i.e., a K_d/K_d^* ratio > 1, see eq 4).

Significance of Et-1 Dose−Response Curves in Intact Cells. Interpretation of Et-1 actions in intact cells ultimately requires a knowledge of the fractional site occupancy achieved at the time of the measures. The difficulty is that Et-1 actions are usually assessed under time-limited, complex second-order kinetic conditions. The only way of defining fractional site occupancies under such conditions is numerical simulation. We first simulated a competition experiment. Conditions chosen (confluent monolayers and 15 min association) were those used for measuring the production of inositol phosphates. Figure 11 defines the range of concentrations at which Et-1 is expected to prevent [^{125}I]Et-1 binding. It shows that expected IC_{50} values are highly sensitive to the k_1 value for Et-1 association to receptors. They are insensitive to k_{-1} values (for values < 5×10^{-3}

min^{-1}). This confirms the intuitive notion that, in most biochemical experiments, Et-1 binds and acts under kinetic rather than thermodynamic conditions. The insensitivity to k_{-1} values further suggests that the macroscopic irreversibility introduced by the internalization of Et-1/receptor complexes has probably little influence on the fractional occupancy of the receptor sites achieved in short term experiments, i.e., on the sensitivity of a preparation to Et-1. This conclusion is independent of the actual fate of [^{125}I]Et-1/receptor complexes and the fact that internalized receptors still signal (Chun et al., 1995).

Figure 11 also presents the results of a real competition experiment. It shows that the results could not be fitted by assuming identical rate constants of association of Et-1 and [^{125}I]Et-1 to receptors. They were best fitted by considering that Et-1 association was 2 times slower than [^{125}I]Et-1 association.

Parameters that satisfactorily fitted competition data were finally used to define the fractional site occupancies at different concentrations of Et-1. Figure 11 shows that, for a 15 min association, 500 pM Et-1 is necessary for half-maximum site occupancy. Thus, under time-limited conditions, receptor occupancy requires sub-nanomolar concentrations of Et-1 rather than picomolar concentrations expected from the measured K_d(Et-1) value. The range of concentrations at which receptors are occupied is similar to, but still lower than, that necessary to produce half-maximum activation of phospholipase C (EC_{50} = 2.3 nM, Figure 7).

DISCUSSION

The objective of this study was to define the affinity of Et-1 for bovine ETA receptors expressed in CCl39 fibroblasts. Two major difficulties were encountered.

1. Et-1/receptor complexes dissociate only slowly. The estimated half-life of the complexes is 2.5 h. Under such conditions, incubation times > 20 h are necessary to reach thermodynamic equilibrium. Et-1 binding is never analyzed under true equilibrium conditions for obvious technical reasons, and this may lead to artifactually high apparent K_d values. This difficulty was overcome by analyzing initial rates of [^{125}I]Et-1 binding to receptors. The results indicated a K_d([^{125}I]Et-1) value of 17 pM. An identical value was obtained from a Scatchard analysis of a saturation experiment performed under quasi equilibrium conditions. This value corresponds to the lower range of K_d values reported in the litterature and to the sites that have been described as super-high-affinity sites (Sokolovsky et al., 1992).

2. Binding of ligands that have a nanomolar affinity for their receptors is easily assessed using pseudo-first-order kinetic approximations. For picomolar ligands, pseudo-first-order conditions are only fulfilled under two conditions: (i) at very large [L*$_o$] values ([L*$_o$] $\gg$ [R$_o$]) and (ii) at very low [R$_o$] and [L*$_o$] values ([R$_o$] < K_d and [L*$_o$] < K_d). The second condition is most suitable for binding experiments, but it leads to very slow association kinetics and a very low specific binding component (unless care is taken to use very large volumes of incubation solution) (Jacobs et al., 1975). Other conditions do not obey well-established kinetics of reversible ligands. They are governed by mathematically complex second-order kinetics. A major consequence is that usual approximations that the free and total concentrations of ligand are identical cannot be made. It follows that apparent K_d values, for instance, determined in competition experiments, are artifactually shifted to higher values (Jacobs et al., 1975). They are not anymore representative of real K_d values and can, under extreme conditions, be measures of [R$_o$] rather than of K_d. Simply stated, apparent K_d values, determined from competition experiments, are measures of real K_d values only if [R$_o$] + [L*$_o$] is negligible (eq 4). The results of competition experiments performed both in membrane preparations (Figure 4) and with intact cells (Figure 9) clearly indicated that different [R$_o$] conditions have a large incidence on apparent affinities of Et-1 for ETA receptors. An uncontrolled [R$_o$] condition may lead to erroneous conclusions about the existence of different affinity states of ET receptors and about structure−activity relationships.

Taken together all results of binding experiments suggest the presence in transfected fibroblasts of a single high-affinity state of bovine ETA receptors for Et-1. The K_d value of Et-1/ETA receptor complexes is of the order of 20 pM.

Et-1 is a potent agonist of phospholipase C in transfected fibroblasts (Figure 7). This action requires concentrations of the peptide that are 100 times higher than the K_d value determined previously. An usual explanation for such an observation is that nanomolar actions of Et-1 are mediated by a low-affinity state of the receptor. This study rather shows that because of time-limited second-order rate conditions, sub-nanomolar concentrations of Et-1 are necessary to occupy a significant fraction of high-affinity (picomolar) receptor sites during the time course of most biochemical experiments (Figure 11). Obvious conclusions are (i) that super-high-affinity sites for Et-1 can mediate short term, nanomolar actions of the peptide and (ii) that there is no need to postulate existence of low-affinity (nanomolar) states of ETA receptors to account for nanomolar actions of Et-1. Indeed, we cannot exclude the possibility that in tissues, yet to be discovered nanomolar affinity sites for Et-1 mediate nanomolar actions of the peptide.

It is of interest to note that while 500 pM Et-1 is sufficient to produce half-maximum receptor site occupancy in a typical biochemical experiment (Figure 11), 2.3 nM Et-1 is necessary to half-maximally stimulate phospholipase C activity (Figure 7). The difference could be a trivial consequence of the fact that initial rates of phospholipase C activity were not measured and could emerge if strong desensitization mechanisms limit the function of activated receptors. Desensitization of seven-transmembrane domain receptors is well known to involve a whole hierarchy of mechanisms including receptor phosphorylation and receptor sequestration (Hausdorff et al., 1990). Desensitization of ETA receptors cannot be easily documented due to the slow dissociation of Et-1/receptor complexes. The shapes of the intracellular Ca^{2+} transients induced by Et-1 in aortic smooth muscle cells are, however, highly suggestive of a rapid desensitization of ETA receptors (Marsault et al., 1991).

Some actions of Et-1 are observed at concentrations as low as a few picomolars (Serradeil-Le Gal et al., 1991; Shirakami et al., 1993; Shraga-Levine et al., 1994; Sokolovsky et al., 1994; Journeaux et al., 1994). They are thought to be mediated by picomolar affinity sites (Sokolovsky et al., 1992, 1993a,b). Kinetic data presented in this paper indicate that picomolar actions of Et-1 can only reflect picomolar affinity sites if [R$_o$] < 10 pM and if very long association times are allowed, two conditions that are rarely met. They also

indicate that under conditions prevailing in most biochemical experiments, sub-nanomolar rather than picomolar concentrations of Et-1 are necessary to occupy a significant fraction of receptor sites (Figure 11). Under such conditions, less than nanomolar actions of Et-1 can be generated, but they require that occupancy of only a fraction of the sites is sufficient to produce a maximum response, i.e., the presence of a receptor reserve. It should also be stressed that because of complex receptor trafficking mechanisms (Kenakin, 1995), different intracellular signals generated by the same receptor may exhibit different sensitivities to the same agonist.

Taken together, these suggest that the sensitivity of any preparation to Et-1 is unlikely to be related to the K_d values of Et-1/receptor complexes. Because of time-limited, second-order kinetics, Et-1 acts as a sub-nanomolar rather than a picomolar agonist of ETA receptors. Amplification mechanisms (i.e., a receptor reserve) may then be responsible for high-affinity actions of the peptide. Conversely, desensitization mechanisms may lead to low-affinity actions of the peptide. With such a scheme, the sensitivity of any preparation to Et-1 has to be viewed as a cellular property rather than as a receptor property. The observation that in brain capillary endothelial cells inhibition of protein phosphatase 1 with calyculin A sensitizes up to 100-fold the Na^+, K^+, $2Cl^-$ cotransporter to the stimulating action of Et-1 (Vigne et al., 1995) is fully consistent with this hypothesis.

The functional consequence of high-affinity (and irreversible) binding is not obvious. It is usually believed that irreversible binding of Et-1 determines long-lasting and irreversible contractile actions of the peptide. Our previous analysis of the contractile action of Et-1 on rat aortic strips indicated that this is not true and that irreversibility of binding and irreversibility of actions are not related (Marsault et al., 1991, 1993). Another advantage of high-affinity binding could be to promote a high potency of the peptide. In fact, this study suggests that any ligand of ETA receptors that would bind more irreversibly than Et-1 would not be more potent than Et-1. This is simply because a slow rate of dissociation of the receptor/ligand complex imposes kinetic conditions that set a lower limit to the potency of a ligand (Figure 11). A last advantage for superhigh binding affinities could be to promote stoichiometric binding conditions which arise whenever $[R_o]/K_d > 100$ (Goldstein, 1944; Henderson, 1972). For a K_d(Et-1) value of 20 pM, tight binding is expected at a receptor concentration of 2 nM. Such a receptor density is probably present in tissues such as vessel walls (Frelin and Guedin, 1994). Stoichiometric binding, by limiting the diffusion of Et-1 into tissues, may account for a number of unusual features of Et-1 physiology (Frelin and Guedin, 1994), such as the fact that blood pressure is elevated in Et-1 +/− transgenic mice (Kurihara et al., 1994), for exceedingly low circulating Et-1 levels, and for the fact that receptor antagonists act as clearance antagonists (Fukuroda et al., 1994).

In conclusion, results presented in this paper suggest that bovine ETA receptors have a single picomolar affinity state for Et-1. For kinetic reasons, however, the potency of Et-1 is only remotely related to the real affinity of the peptide for its receptor, and nanomolar actions of Et-1 can be mediated by picomolar affinity sites. High-affinity binding is probably important for favoring short range (mainly autocrine) actions of the peptide.

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