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Receptor Kinetics Differ for Endothelin-1 and Endothelin-2 Binding to Swiss 3T3 Fibroblasts

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The equilibrium binding, kinetics of ligand-receptor interactions, and biological activity of endothelin-1 and -2 have been studied in Swiss 3T3 fibroblasts. Scatchard analyses of saturation binding data for ET-1 and -2, performed at 4°C to prevent internalization of the occupied receptor, revealed similar affinity constants and numbers of binding sites for endothelin-1 and -2. Experiments designed to determine ligand-induced effects on ^{45}Ca efflux demonstrated no qualitative or quantitative differences between the two endothelin isoforms. In contrast, kinetic studies resulted in different rates of dissociation for the two isoforms and different extents of dissociation. Specifically, only 40% of the bound [^{125}I]endothelin-1 was dissociated at 4 h following the addition of excess unlabeled ligand, whereas 85-90% of the bound [^{125}I]endothelin-2 was dissociated under the same conditions. Endothelin-1 and -2 also differed in the percent of specific cell-associated ligand bound after a 2 h incubation at 37°C following an initial equilibration at 4°C. The differences in dissociation rates and association or internalization rates at 37°C are the first data that differentiate between the two isoforms. It is suggested that isoform-specific differences in the rate of dissociation from cell surface endothelin receptors influence the level of cell-associated endothelin and may be important in determining physiologic responses in vivo.

Endothelin (ET), a 21 amino acid peptide produced by endothelial cells, is the most potent vasoconstricting peptide known (1). Three separate genes encoding distinct isoforms of endothelin have been identified by Southern analysis of human, rat, and porcine genomic DNA (2). The predicted amino acid sequences of the three endothelin isoforms from the human genome differ by 2 and 6 residues for endothelin-2 and -3, respectively, compared to the 21 amino acids of mature endothelin-1. The cellular sites of production of the individual endothelin isoforms, as well as their sites of action, remain to be elucidated. Northern blot analysis of mammalian cells and tissues indicates that ET-1 message is found primarily in endothelial cells (2) whereas ET-3 message has been detected in a wide range of tissues (3). No comparable data exist for ET-2 expression. Isoform-specific receptor binding studies have identified endothelin receptors in human and murine fibroblast cell lines (4), as well as in chick cardiac membranes (5) and rat lung membranes (6). Data obtained from binding isotherms and chemical crosslinking studies with [^{125}I]endothelin isoforms, followed by SDS-PAGE analysis, imply that a single receptor binds ET-1 and ET-2 with roughly comparable affinity, and a second lower affinity receptor preferentially binds ET-3 (5,6).

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One of the hallmarks of endothelin action is a long duration of biological activity. Contraction of porcine coronary strips in response to high doses of ET-1 persisted for at least 20 min following washout of endothelin (1). Similar results were reported in dog coronary arteries (7), feline middle cerebral arteries (8), canine basilar arteries (9), and in renal arteries from normal and spontaneously hypertensive rats (10). The duration of response to endothelin may be due in part to its extremely slow dissociation from its receptor. For example, [^{125}I]labeled ET-1 bound to human vascular smooth cells was only 50% dissociable following a 24 h incubation at room temperature with excess unlabeled ET-1 (11) and similar experiments performed at 37°C in rat smooth muscle cells resulted in only 15% dissociation after 2 h (12). Since both experiments were performed at temperatures that could allow internalization of occupied endothelin receptors, it is not clear from these studies whether the slow rate of dissociation is due to internalization and subsequent recycling of intact or degraded endothelin to the cell surface, or whether it is an intrinsic function of endothelin bound to the receptor. The latter interpretation is supported by the observation that in rat cardiac membranes only 30% of bound ET-1 was dissociated by 2 hours following the addition of excess cold ligand (13). In order to explore whether slow dissociation of endothelin from its receptor is unique to endothelin-1, a number of binding experiments were performed on Swiss 3T3 fibroblasts, a cell type shown to respond to endothelin with increases in intracellular Ca^{2+} (3). The data presented here suggest that ET-1 and ET-2 bind to the same receptor, but that slow dissociation of endothelin bound to this receptor is unique to ET-1.

Materials and Methods

Materials

(3-[^{125}I]-iodotyrosyl endothelin-1 and -2, specific activity 2200 Ci/mmol, were purchased from Amersham (Arlington Heights,IL). Endothelin -1, -2, and -3 were from American Peptides Corp, Santa Clara, CA. Swiss 3T3 fibroblasts were obtained from American Type Culture Collection, Rockville, MD, and grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% calf serum, penicillin (100 U/ml), streptomycin (100 ug/ml), and gentamicin (100 ug/ml) (Gibco, Grand Island, NY).

Equilibrium Analysis

Swiss 3T3 fibroblasts were plated in 60 mm^2 tissue culture dishes and allowed to reach confluency before use. Prior to addition of labeled and unlabeled endothelins, the cells were incubated at 4°C for 30 min, washed twice with PBS and incubated in DMEM, 25 mM Hepes pH 7.4, 0.15% w/v gelatin, 25 mM $MnCl_2$ and increasing concentrations of [^{125}I]labeled ET-1 (1-10 nM). Non-specific binding was determined at each concentration of labeled isoform by a parallel incubation in the presence of unlabeled ET of the corresponding isoform (10 uM) and never exceeded 5% of the total cell-associated radioactivity. All data points were measured in triplicate. Cells were incubated with labeled ligands in the presence or

absence of unlabeled ligands for at least 3 hr at 4°C with gentle shaking. Incubations were terminated by aspiration of the binding medium, followed by 2 washes with ice-cold phosphate-buffered saline (PBS) pH 7.4. Triton X-100 (0.5 ml, 0.5% v/v) was added to the monolayers for at least 5 min to solubilize cells and associated radioactivity. The medium, cell washes and the solubilization mixture were counted in a Beckman gamma counter. The cell number of representative dishes was determined following trypsinization of the cells.

Determination of ^{45}Ca Efflux

Cells were loaded to isotopic equilibrium with ^{45}Ca by incubating the 3T3 cells on 22 x 22 mm coverslips in DMEM plus FCS containing 20 μCi/ml ^{45}Ca for 4 hours. The cells were washed in 20 ml of physiological salt solution (PSS: $NaCl_2$, 140 mM; KCl, 5 mM; $CaCl_2$, 1.5 mM; $MgCL_2$, 1.0 mM; HEPES, 5mM; D-glucose, 10 mM; EDTA, 20 μM; pH 7.4 at 25°C) at 37°C for 1 min before being transferred at 2 min intervals at 37°C to a series of ten 25 mm^2 petri dishes each containing 4 ml of a calcium free PSS containing 2 mM EGTA. ET-1 or ET-2 were added at a concentration of 100 nM at 12-14 min of efflux. At the end of the efflux period the cells, on the coverslip, were placed into 2 ml of a 5 mM EDTA solution to remove the cells from the coverslip and to remove the ^{45}Ca from the cells. The combined calcium content of both the cells and the efflux solutions was determined by liquid scintillation spectrometry. The initial calcium content of the cells was determined by adding the total amount of calcium lost per min throughout the washes to the final calcium content of the cells. The results are expressed as the efflux coefficient (min^{-1}) which was calculated as one half of the ^{45}Ca efflux during a given two minute interval, divided by the average ^{45}Ca remaining within the cells during the same interval.

Dissociation of Bound Labeled Endothelin

3T3 fibroblasts were maintained as described above. Labeled ET-1 and ET-2 were added to monolayers at a final concentration of 5 or 20 pM and allowed to come to equilibrium for 3 h at 4°C. Dissociation of bound labeled endothelins was induced by the addition of excess unlabeled endothelin (10 μM) of the corresponding isoform. Cell-associated endothelin was measured at various times following the addition of unlabeled endothelin as described above. Non-specific binding was determined as described above.

Temperature Shift Studies

Cells were incubated at 4°C with labeled ET-1 or ET-1 for 3 hr. Incubation dishes were then shifted to 37°C for the indicated times and harvested as described above. Control incubations were maintained at 4°C for comparable lengths of time.

Data Analysis

Equilibrium analyses were plotted according to the method of Scatchard (12) using the Enzfitter software program (Biosoft, Milltown, NJ). Dissociation experiments were normalized relative to the level of bound endothelin measured immediately prior to the addition of excess unlabeled endothelin. Temperature shift experiments were normalized to control dishes held at 4°C for matched lengths of time.

Results

Equilibrium binding of endothelin to Swiss 3T3 fibroblasts was performed at 4°C. At this temperature, specific binding of ET-1 and ET-2 reached steady state by 3 h, and was 85-90% releasable by a 2 min exposure to pH 2.5 (data not shown). Scatchard analysis of the

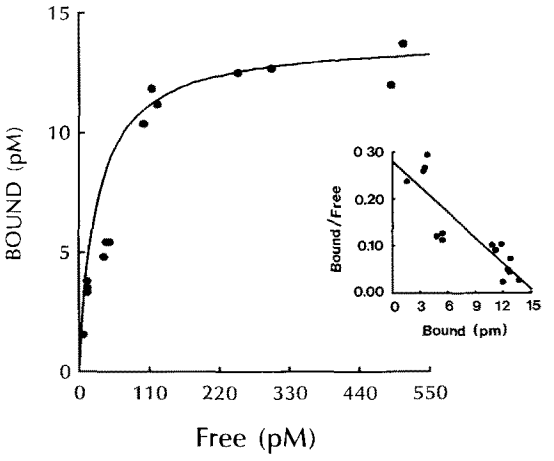


Fig. 1. Saturation Binding Data and Scatchard Analysis for ET-1 Binding to Swiss 3T3 Cells. Cells were incubated with increasing concentrations of labeled ET-1 (Spec Act 2200 Ci/mmol) for 3 h at 4°C. Non-specific binding was determined by incubating with 10 μM unlabeled ET-1. This is a representative experiment where data points were obtained in triplicate and the experiment was repeated four times.

equilibrium saturation binding data imply that ET-1 (Fig. 1) and ET-2 (data not shown) bind to a single class of receptors with half-maximal binding affinities of 22.5 +/- 5.0 pM and 24.2 +/- 3.3 pM, respectively. At apparent saturating concentrations of ET-1 or ET-2 there are 84,200 +/- 8,900 and 56,500 +/- 1,700 sites/cell, respectively (Table I). The apparent B_{max} values are not statistically different. Unlabeled ET-1 or ET-2 compete with equal affinities for binding of both labeled ligands (data not shown). No specific binding sites for ET-3 were detectable up to 100 pM under the same experimental conditions used for ET-1 or ET-2.

In order to measure calcium efflux, 3T3 cells were pre-loaded with ^{45}Ca, transferred to a calcium free solution and the rate of efflux of ^{45}Ca determined over a 20 min period. When the cells were exposed to 100 nM ET-1 or ET-2 between 12-14 min, the rate of calcium efflux increased as determined by an increase in the calcium efflux coefficient (Fig. 2).

Dissociation of bound, labeled endothelin isoforms was measured following a 3 h, 4°C association phase with 50 pM labeled ligand. Addition of a 1000-fold excess of unlabeled

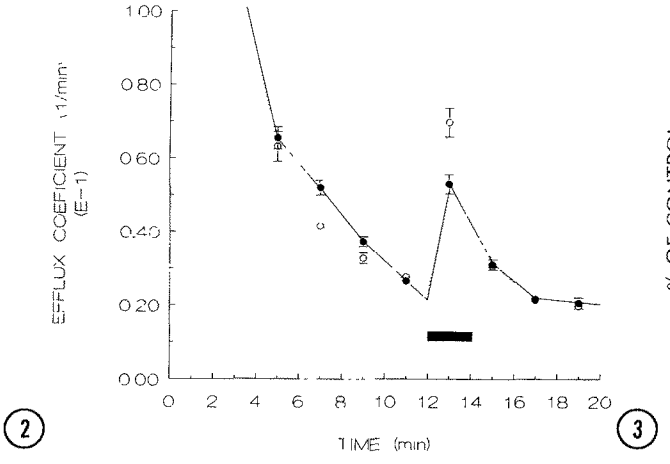
Table 1. Binding Data for Endothelin-1 versus Endothelin-2 on Swiss 3T3 Fibroblasts [1]

ET-1 (n=4)		ET-2 (n=4)	
K_d (pM)	B_{max} (sites/cell)	K_d (pM)	B_{max} (sites/cell)
22.5 ± 5.0	84,200 ± 8,900	24.2 ± 3.3	56,500 ± 1,700

[1] Data were derived from a Scatchard analysis of saturation binding data obtained as described in Methods.

ET-1 to incubations containing labeled ET-1 resulted in a slow and limited dissociation of bound [^{125}I]ET-1 (Fig. 3). After 4 h of incubation with excess unlabeled ET-1, 48% of ET-1 was dissociated. In contrast, addition of a 1000-fold excess of unlabeled ET-2 to cells first incubated with [^{125}I]ET-2 (50 pM) resulted in 82% dissociation of bound labeled ligand by 4 h (Fig. 3). The rates of dissociation of both labeled ligands were multiexponential with a rapid phase occurring for ET-2 in the first 1 h, followed by a slower phase over the next 3 h and an initial lag for ET-1 dissociation in the first 1 h, followed by a somewhat faster phase over the next 3 h.

The quantity of cell-associated endothelin isoforms also differed when binding was assessed at 37°C following pre-equilibration at 4°C. In this "temperature-switch" experiment, labeled endothelin -1 or -2 (5 pM or 20 pM) was allowed to equilibrate with cells at 4°C for 3 h, then transferred to 37°C for 2 h. Specific cell-associated ligand was quantitated at various time points following the temperature change. Fig. 4 is a representative set of ligand binding data obtained using this protocol and 5 pM [^{125}I]-ET-1. The binding of both ET-1



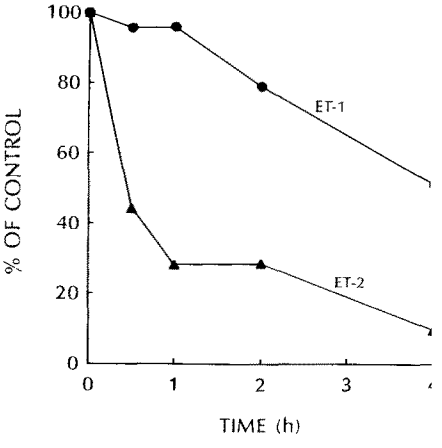


Fig. 2. The Effects of ET-1 and ET-2 on the Efflux of ^{45}Ca from Swiss 3T3 Cells. Confluent monolayers of cells on coverslips were loaded to isotopic equilibrium with ^{45}Ca, exposed to 100 nM ET-1 (●) or ET-2 (○) for 2 min, and the rate of ^{45}Ca efflux into a calcium free physiological saline solution containing 2 mM EGTA determined as described in the Methods Section. The graphed data were obtained in quadruplicate using individual coverslips of cells for each calcium efflux time course. The data are the mean ± SEM of the four different coverslips of cells.

Fig. 3. Comparison of the Dissociation of ET-1 and -2 from 3T3 Fibroblasts. Swiss 3T3 cells were incubated with 50 pM labeled ligand for 3 h at 4°C. The monolayers were washed with cold PBS and incubated with 10 μM unlabeled ligand, ET-1 (●) or ET-2 (▲) for various times. Non-specific binding was determined with 10 μM unlabeled ligand present in both association and dissociation phases. The data are the mean from four experiments with ET-1 where individual data points were obtained in triplicate and the mean for three experiments with ET-2 where individual data points were obtained in triplicate. The SEM is less than the size of the graph symbol.

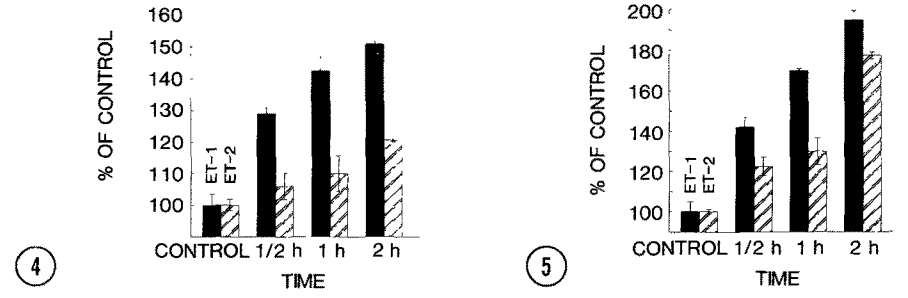


Fig. 4. **Internalization of ET-1 and ET-2 in 3T3 Cells.** Swiss 3T3 cells were incubated at 4°C for 3 h with 5 pM of [^{125}I]ET-1 (solid bars) or [^{125}I]ET-2 (cross-hatched bars). The cells were transferred to 37°C for various times and harvested as described in Materials and Methods. Each bar represents the mean ± SEM for four experiments where each data point was obtained in quadruplicate.

Fig. 5. **Internalization of ET-1 and ET-2 in 3T3 Cells.** Swiss 3T3 cells were incubated at 4°C for 3 h with 20 pM of ^{125}I-ET-1 (solid bars) or ^{125}I-ET-2 (cross-hatched bars). The cells were transferred to 37°C for various times and harvested as described in Materials and Methods. Each bar represents the mean ± SEM for four experiments where each data point was obtained in quadruplicate.

and ET-2 increased by 30 min following the shift from 4°C to 37°C relative to a control incubation maintained at 4°C. Binding of both ligands continued to increase for the maximal time observed, 2 h at 37°C. At all time points the increase in binding of ET-1 relative to a control incubation maintained at 4°C was larger than the increase in ET-2 binding relative to a 4°C control. Similar results were seen with 20 pM ET-1 or ET-2 (Fig. 5).

Discussion

The current study demonstrates that endothelins -1 and -2 bind to cell surface receptors on Swiss 3T3 fibroblasts with similar affinities and that the number of isoform-specific cell-surface receptors is similar, and that unlabeled ET-1 and ET-2 compete with equal affinities for binding of both labeled ligands. Thus, several binding parameters determined at apparent equilibrium suggest that the two ligands bind to a common receptor. These data are consistent with published reports which show that when [^{125}I]ET-1 and -2 are chemically crosslinked to chick cardiac or rat lung membranes and analyzed by SDS-PAGE (5,6), the radioactively labeled proteins have similar mobility. ET-3, in contrast, appeared to label a smaller protein in these studies. Under the conditions used in the present study, binding of ET-3 to 3T3 fibroblasts was not detectable within the same concentration range as used for ET-1 and -2 implying either too few receptor sites for detection or receptor sites of much lower affinity. These data suggest that Swiss 3T3 fibroblasts have only one type of endothelin receptor, which recognizes ET-1 and ET-2 with a much greater affinity than that for ET-3.

The present study was designed to examine equilibrium binding at 4°C where internalization is inhibited. Thus the data represent the number of cell surface receptors as well as the affinity of the ligand for these cell-surface receptors. The $K_{d,apparent}$ determined for ET-1 and ET-2 under these conditions was approximately 23 pM. In contast, the equilibrium binding of endothelin to rat smooth muscle cells (15) or 3T3 fibroblasts (4) at 37°C involves a more complicated experimental situation where not only cell surface-ligand interactions are involved, but possible internalization of receptor-bound ligand. For example, in a study by Ohnishi and coworkers (4), the $K_{d,apparent}$ determined for ET-1 and ET-2 binding to 3T3 cells at 37°C was approximately 500-520 pM. There is a 20-fold difference in affinity between the values obtained in the present study done at 4°C and those of Ohnishi-Suzaki done at 37°. Other than the temperature difference, it is possible that either the synthetic ET used in the present study is more potent or the 3T3 cells are more responsive.

The similarity in the ^{45}Ca efflux in response to ET-1 or ET-2 imply that these ligands are working through similar receptor signalling mechanisms, but provide no information about whether the ligands bind to the same receptor. In fact, the efflux response data were obtained at a saturating concentration of ligand where subtle receptor-dependent differences would not be observed.

Despite the fact that the equilibrium binding data suggest that the two ligands interact with similar affinities to an equal number of cell-surface receptors, and that they elicit similar biological responses at saturating concentrations of ligand, ET-1 and ET-2 dissociate from the receptor(s) at significantly different rates and to different extents. These data imply that if these ligands bind to the same receptor, then the substitution of only two amino acids is responsible for a dramatic alteration in the kinetics of endothelin isoform binding. This suggests that either the substituted positions, amino acids 6 and 7, are in intimate contact with the receptor when endothelin is bound, or the substitution of a Trp for Leu^6 and a Leu for Met^7 significantly alters the tertiary structure of ET-2 relative to ET-1, such that the ligand-receptor interaction kinetics are altered.

The differences in the dissociation rates observed at 4°C reflect only binding to the cell surface receptor. The increase in cell-associated binding of ET-1 relative to ET-2 when the incubation temperature of the cells is changed from 4°C to 37°C suggests that the difference in dissociation rates might persist in the more complex situation that exists at elevated temperatures. This increase in binding of both ligands seen upon shifting the cells from 4°C to 37°C could be explained by several hypotheses. The first is that there is a pool of receptors that rapidly becomes available when fibroblasts are shifted from 4°C to 37°C. A similar situation has been demonstrated in cultured cardiocytes where a pool of intracellular endothelin receptors shifts to the plasma membrane under ischemic conditions (16). An

alternative explanation is that endothelin receptors, like the receptors for LDL (17), transferrin (18), insulin (19), and EGF (20-28), internalize with bound ligand, release the ligand and then recycle to the plasma membrane where they are available to bind new ligand.

The differences in the dissociation rates of ET-1 and ET-2 from cell-surface receptors at 4°C and the differences in the association rates or internalization rates at 37°C are the first data that differentiate between the two isoforms, which otherwise differ only slightly in all biological parameters currently measured. The difference in rates of binding of endothelin isoforms suggests that the isoforms may have distinct and different biological functions, and thus may offer the first clue as to why genes for two such similar endothelin isoforms persist in species ranging from rat to man (2).

In summary, the results presented here demonstrate that 3T3 fibroblasts have receptors for ET-1 and ET-2, but not ET-3 and that the receptor affinities and numbers, measured at 4°C to limit binding to cell-surface receptors, are similar for both ET-1 and ET-2. The addition of saturating concentrations of the two ligands do not produce significantly different effects on ^{45}Ca efflux rates from the cells. The rate and extent of dissociation of ET-1 from cell-surface receptors are similar to those previously reported for other cell types and membranes. In contrast, the dissociation of ET-2 is more rapid and proceeds to a greater extent than that of ET-1. Given the difference in binding between the two endothelin isoforms at 37°C, it is likely that the observed difference in dissociation rates at 4°C also holds at higher temperature.

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