CHARACTERIZATION OF RECOMBINANT ENDOTHELIN RECEPTOR TYPE B OVEREXPRESSED IN CHINESE HAMSTER OVARY CELLS

NOBUAKI ETOH, MOTOHIRO KONDOH, JUNJI OHNISHI, KAZUO MURAKAMI and HITOSHI MIYAZAKI
Institute of Applied Biochemistry, Gene Experiment Center, University of Tsukuba, Ibaraki 305, Japan

ABSTRACT

The recombinant B type of endothelin (ET) receptor (ET_B receptor) was expressed at high levels in Chinese hamster ovary (CHO) cells. The recombinant ET_B receptor possessed biochemical characteristics of the endogenous ET_B receptor with regard to affinities, specificities for ET isopeptides, and molecular mass. In the transfected CHO cells, high concentrations of ET (10^{-10} M $\leq$) caused a rapid, transient increase in intracellular free Ca²⁺ concentrations ([Ca²⁺]_i) followed by a lower, sustained phase. In contrast, low concentrations of ET ($\leq 10^{-11}$ M) induced only the sustained [Ca²⁺]_i phase. Removal of extracellular Ca²⁺ and the addition of nicardipine, an antagonist of L-type Ca²⁺ channels, completely blocked and significantly decreased the sustained [Ca²⁺]_i response, respectively. These results in conjunction with the findings that ET-induced inositol triphosphate production was observed at only high concentrations of ET (10^{-10} M $\leq$) suggest that the ET_B receptor preferentially couples with the induction of Ca²⁺ channel opening at low ligand concentrations ($\leq 10^{-11}$ M), whereas that the receptor causes both activation of phospholipase C and Ca²⁺ channel opening at high ligand concentrations (10^{-10} M $\leq$). Rapid internalization of the ET-ET_B receptor complex with a half time of 15 min was detected by affinity labeling, presenting the necessity for studying correlation between the internalization and ET_B receptor-mediated generation of intracellular signals. Finally, this study demonstrates the usefulness of our transfected CHO cells stably expressing the recombinant ET_B receptor, with which signal transduction pathways through this receptor and internalization of the ET-ET_B receptor complex can be analyzed.

Endothelin (ET)-1 is a 21-amino acid peptide initially isolated from culture media of porcine aortic endothelial cells and shown to be one of the most potent vasoactive peptides known today (32). In addition to ET-1, different isoforms of ET, named ET-2 and -3, are now recognized (6, 18). Endothelins mediate their biological actions by binding to specific cell surface receptors on target tissues (14, 20). In mammals, two types of ET receptors, named ET_A and ET_B, have been identified based on molecular biological and pharmacological studies

(1, 3, 11, 19) although other subtypes such as ET_C have been found in *Xenopus laevis* and chick (8, 28). Both the ET_A and ET_B receptors include seven membrane-spanning domains, characteristic of G-protein-coupled receptors. The ET_A receptor binds ET-1 and -2 with a higher affinity than ET-3, whereas the ET_B receptor binds all three isopeptides with a similar high affinity.

Molecular cloning of the cDNA for ET receptors has demonstrated that both these receptor types are linked to the inositol/Ca²⁺ second messenger pathways (1, 2, 11, 19); i.e. the binding of ligands to the receptors activates phospholipase C (PLC) resulting in the formation of inositol triphosphate (InsP₃), which raises cytosolic free Ca²⁺ concentrations ([Ca²⁺]_i). Signal transduction pathways induced by ET via the ET_A receptor have been well

Abbreviations: SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; EGTA, ethylene glycol bis(b-aminoethyl ether)-N,N,N',N'-tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

characterized in various cultured cells (21, 24, 25, 30, 31). However, little information is available concerning ET_B receptor-mediated signal transductions, especially ET-induced Ca²⁺ mobilization, because of the lack of suitable cultured cells expressing sufficient levels of the ET_B receptor. For example, even in endothelial cells, a major source of the ET_B receptor, its concentration is only 40 fmol per mg protein, being at least 10-fold lower than that of the ET_A receptor in smooth muscle cells (23). These facts indicate that stably transfected cells expressing high levels of the ET_B receptor are essential to facilitate understanding of ET_B receptor-mediated signal transduction pathways in detail.

The initial binding of ligands to their receptors is often followed by endocytosis and processing of the ligand-receptor complex (15, 29). Some investigators have demonstrated a striking correlation between internalization of angiotensin II (AII)-receptor complexes and the generation of second messengers such as InsP₃ and diacylglycerol (4, 5, 27). Resink *et al.* (17) have reported that ET-induced vasoconstriction mediated by the ET_A receptor is attenuated in isolated endothelium-denuded blood vessels by adding inhibitors of transglutaminase which is necessary for internalization. Moreover, Marsault *et al.* (12) have recently demonstrated that internalization and recycling of the ET_A receptor determine sustained contractile responses to ET in the rat aorta. Therefore, to study ET_B receptor-mediated signal transduction pathways, it is of key importance to examine whether ET-ET_B receptor complexes are internalized.

In the present study, we expressed high levels of rat ET_B receptor cDNA in Chinese hamster ovary (CHO) cells using dihydrofolate reductase (*dhfr*) gene amplification. In these stably transfected CHO cells, we investigated (a) ET_B receptor-mediated inositol phosphate/Ca²⁺ second messenger pathways in detail, focusing on ET-induced Ca²⁺ mobilization, and (b) internalization of ET-ET_B receptor complexes. The existence of cells overexpressing the ET_B receptor should provide a powerful means to understand the mechanism of ET-induced biological actions via the ET_B receptor *in vivo*.

EXPERIMENTAL PROCEDURES

Materials

CHO cells (DXB-11 strain), which were *dhfr*-deficient, were obtained from Upjohn Company (Kala-

mazoo, MI, U.S.A.). An expression plasmid was constructed using the ET_B receptor cDNA previously described (19). Cell culture media, balanced salt solutions, non-dialyzed fetal calf serum (FCS), methotrexate (MTX), and dansylcadaverin were purchased from Sigma. Dialyzed FCS was from Cell Culture Laboratories (Cleveland, OH, U.S.A.). ¹²⁵I-ET-1 and -3 (~2,000 Ci/mmol) were obtained from Amersham International plc (Bucks, U.K.). ET-1 and -3 were from Peptide Institute (Osaka, Japan) and disuccinimidyl suberate (DSS) was purchased from Pierce Chemical (Rockford, U.S.A.).

Cell Culture

CHO cells which were *dhfr*-deficient were grown in Nutrient mixture F-12 Ham medium (Ham F-12) supplemented with 10% FCS, 20 mM L-glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin. Transfected CHO cells were propagated in Dulbecco's modified Eagle's medium (DMEM) supplemented with L-glutamine, the antibiotics listed above, and 10% dialyzed FCS (selection medium). Cultures were maintained at 37°C in a humidified atmosphere consisting of 95% air, 5% CO₂ and routinely passaged by trypsinization.

Construction of Expression Plasmid, DNA Transfection, and MTX Amplification

A 2.0-kb *Xba* I fragment containing the entire coding sequence for the rat ET_B receptor was obtained from plasmid pRETR-7 (19) and digested with *Rsa* I located at 83 bp upstream from the initiation codon ATG. The resulting 1.8-kb *Rsa* I-*Xba* I fragment was blunt-ended, ligated with *Bam* HI linkers, then digested with *Bam* HI. Thereafter, the fragment was cloned into the *Bam* HI site of the expression vector pSVD originally constructed for renin expression (16). The resulting plasmid pSVD_{ETBR} was transfected into *dhfr*-deficient CHO cells by calcium phosphate coprecipitation. Twenty CHO transfectants expressing the *dhfr* phenotype were isolated by propagation in selection medium. Out of these clones, five clones exhibiting high concentrations of ¹²⁵I-ET-1 binding sites (2.5 to 4.9 × 10⁵ sites per cell) were chosen; these concentrations were nearly equivalent to those of mouse Swiss 3T3 fibroblasts, which express the highest level of the ET_A receptor currently known (~3 × 10⁵ sites/cell) (24). These clones were then grown in stepwise increasing concentrations of

MTX (100, 200, 300, 400, 500, 600, and 700 nM) to amplify the ET_B receptor cDNA. After MTX selection, the highest producer of recombinant ET_B receptor molecules (1.5×10^6 sites/cell) was obtained and this clone was subsequently studied.

Equilibrium Binding and Competitive Binding Studies

For equilibrium binding studies, cells in 24-well plastic dishes ($\sim 1 \times 10^4$ cells/well) were washed twice with 0.5 ml ice-cold buffer A consisting of Hanks' balanced salt solution (in mM: NaCl, 137; KCl, 5.4; KH₂PO₄, 0.44; Na₂HPO₄, 0.33; MgSO₄, 0.40; MgCl₂, 0.5; CaCl₂, 1.25; and NaHCO₃, 4.0) buffered with 20 mM 4-(2-hydroxyl)-1-piperazine-ethanesulfonic acid (Hepes), pH 7.4. Each well was incubated with increasing concentrations (5–400 pM) of ¹²⁵I-ET-1 or -3 in the presence and absence of 100 nM unlabeled ET-1 or -3, respectively, for 3 h at 4 or 37°C in a total volume of 0.2 ml binding assay buffer consisting of buffer A containing 0.1% crystallized bovine serum albumin (BSA). The attached cells were then washed twice with buffer A to remove free ligands, dissolved in 1 ml of 1 N NaOH, transferred to a test tube, and the radioactivity was counted. For competitive binding studies, after washing twice with 0.5 ml buffer A, confluent cells in 24-well dishes ($\sim 1.5 \times 10^5$ cells/well) were incubated for 2 h at 37°C in 0.2 ml binding assay buffer with 50 pM ¹²⁵I-ET-1 in the presence of increasing concentrations (10^{-12} – 10^{-6} M) of unlabeled ET-1 or -3. Under these conditions, ¹²⁵I-ET-1 binding reached a plateau after 2 h.

Affinity Labeling

Confluent cells in 6-well plates were incubated in 1 ml binding assay buffer for 2 h at 37°C with 100 pM ¹²⁵I-ET-1 or -3 in the presence and absence of 100 nM unlabeled ET-1 or -3, respectively. Thereafter, the attached cells were washed twice with 1 ml phosphate-buffered saline (PBS: 10 mM phosphate, 120 mM NaCl, pH 7.4) to remove free ligands, then the covalent cross-linking reagent DSS, dissolved in dimethyl sulfoxide, was added to a final concentration of 1 mM. Cross-linking was performed for 60 min at 4°C and terminated by washing twice with 0.5 ml of buffer A. To study internalization of ET-ET_B receptor complexes, cells were reacted with radioligands at 4 or 37°C for the indicated periods of time shown in the figures and the cross-linking reaction proceeded for 60 min at

4°C. Cells were suspended in 100 μ l sodium dodecyl sulfate (SDS)-gel sample buffer (62.5 mM Tris-HCl, 10% glycerol, 2% SDS, 0.01% bromophenol blue, pH 6.8, without a reducing reagent) and heated for 3 min at 100°C. Samples were separated by SDS-polyacrylamide gel electrophoresis (PAGE) using 1.0-mm thick gels (10%). The gels were then dried and exposed to Fuji medical X-ray film with intensifying screen at –70°C. The intensity of affinity-labeled bands was measured using a Bio Image densitometric analyzer (Milligen/Biosearch).

Measurement of InsP₃ Production

Confluent cells in 35-mm dishes ($\sim 1 \times 10^6$ cells/well) were prelabeled with myo-[2-³H]inositol (4 μ Ci/ml) in DMEM containing 0.3% BSA for 24 h and stimulated by the indicated concentrations of ET-1 for 1 min as shown in Fig. 5A. The reaction was terminated with ice-cold perchloric acid. The neutralized perchloric acid extract was analyzed for inositol phosphates, using Dowex columns as described (26).

Measurement of [Ca²⁺]_i

Intracellular Ca²⁺ concentration ([Ca²⁺]_i) was determined with the Ca²⁺-sensitive dye fura-2. Confluent cells ($\sim 1 \times 10^7$ cells) grown on 100-mm diameter dishes were trypsinized. The trypsin action was terminated by soybean trypsin inhibitor, then the harvested cells were washed twice with buffer B (in mM: NaCl, 140; KCl, 4; MgCl₂, 1; CaCl₂, 1.25; NaH₂PO₄, 1; Hepes, 5; glucose, 11; 0.1% BSA, pH 7.4), and incubated with 4 μ M fura-2/AM, dissolved in dimethyl sulfoxide, in the same buffer for 50 min at 20°C. The resultant fura-2-loaded cells were washed twice with buffer B without 0.1% BSA, and resuspended in 10 ml of the same buffer. About 10⁶ cells/ml were used in each experiment. The fluorescence was measured with a CAF-100 spectrofluorometer (Japan Spectroscopy, Tokyo, Japan) with excitation at 340 and 380 nm and emission at 500 nm. Cell suspensions in cuvettes were constantly stirred and maintained at 30°C during the measurement of fluorescence.

RESULTS

Biochemical Characterization of Recombinant ET_B Receptor

Biochemical characteristics of recombinant ET_B receptor, such as affinities and specificities for ET

Fig. 1 Binding of ^{125}I -ET-1 to CHO cells expressing the rat ET_B receptor at 37°C and 4°C . A and C: Representative saturation isotherms of ^{125}I -ET-1 binding to the transfected CHO cells at 37°C and 4°C , respectively. B and D: Scatchard analyses of ^{125}I -ET-1 binding to the CHO cells obtained from the saturation data from A and C, respectively; the dissociation constant $K_\text{d} = 45$ pM, the number of binding sites $B_\text{max} = 1.82 \times 10^6$ sites/cell at 37°C and $K_\text{d} = 54$ pM, $B_\text{max} = 8.8 \times 10^5$ sites/cell at 4°C

isopeptides and molecular mass, were examined. Fig. 1A represents a typical saturation isotherm for ^{125}I -ET-1 binding to the transfected cells. The data, expressed as a Scatchard plot, yielded an apparent straight line indicative of one class of binding sites (Fig. 1B). The dissociation constant (K_d) value determined by three different experiments was 75 ± 30 pM. A similar result, with K_d of 67 ± 20 pM ($n = 3$), was obtained when ^{125}I -ET-3 was used as the ligand (data not shown). These K_d values were consistent with those (10^{-10} – 10^{-11} M) previously reported using various kinds of cultured cells (20). The numbers of the binding sites (B_max) were $1.50 \pm 0.37 \times 10^6$ and $1.42 \pm 0.47 \times 10^6$ sites/cell ($n = 3$) for ET-1 and -3, respectively. Assessment of ET_B receptor density will be discussed below in detail because we found that equilibrium binding studies performed at 37°C do not

accurately represent the number of cell surface receptors, due to internalization of ET- ET_B receptor complexes. The ligand specificity of the recombinant receptor was investigated by means of competitive inhibition, in which the cells were incubated with 50 pM ^{125}I -ET-1 in the presence of increasing concentrations of unlabeled ET-1 or -3. In conclusion, the recombinant ET_B receptor exhibited nearly identical affinity for both ET isopeptides (data not shown).

To determine the molecular mass of the recombinant receptor, cells were affinity-labeled by cross-linking with ^{125}I -ET-1 or -3 via the bifunctional reagent DSS. Affinity-labeled proteins were separated by SDS-PAGE without a reducing reagent, then autoradiographed. As shown in Fig. 2, one major band corresponding to a molecular mass of 58 kDa appeared in both samples labeled with ^{125}I -

Fig. 2 Affinity labeling of ^{125}I -ET-1 and -3 with the ET_B receptor expressed in CHO cells. The transfected CHO cells were incubated with 100 pM ^{125}I -ET-1 (lanes A and B) or ^{125}I -ET-3 (lanes C and D) in the presence (lanes B and D) and absence (lanes A and C) of 100 nM unlabeled ET-1 or -3. Approximately 30 μg of protein were loaded in each lane of the gel. Arrow showing 58 kDa indicates a specific band for ET. On the right, molecular mass standards

ET-1 (lane A) and -3 (lane C). The presence of an excess concentration (100 nM) of unlabeled ET-1 (lane B) and -3 (lane D) during the incubation of the cells with ^{125}I -ET-1 and -3, respectively, abolished the appearance of this band, indicating the specificity of the band for ET. Subtracting the molecular masses of the radioligand and DSS, that of the recombinant receptor was approximately 55 kDa, being in good agreement with that of purified bovine ET_B receptor (9).

Effect of ET on $[\text{Ca}^{2+}]_i$

The ET-induced Ca^{2+} mobilization in the transfected cells was examined by measuring the changes in $[\text{Ca}^{2+}]_i$ using fura-2 as a Ca^{2+} indicator. Addition of 10^{-8} M ET-1 or -3 caused a rapid, transient increase in $[\text{Ca}^{2+}]_i$ which peaked at 15 s followed by a lower, sustained phase (Fig. 3A). In contrast, ET-1 or -3 at 10^{-11} M induced a small and sustained increase in $[\text{Ca}^{2+}]_i$, but not an initial, transient phase (Fig. 3B). The Ca^{2+} mobilization at various concentrations of ET-1 or -3 (10^{-12} – 10^{-7} M) indicated that ET at high concentrations (10^{-10} – 10^{-7} M) evoked a

Fig. 3 Increase in $[\text{Ca}^{2+}]_i$ in response to ET-1 and -3. The transfected CHO cells loaded with fura-2 were stimulated with 10^{-8} ET-1 and -3 (A) and 10^{-11} M ET-1 and -3 (B) in Ca^{2+} -replete buffer, and $[\text{Ca}^{2+}]_i$ was measured by dual-wavelength fluorimetry as described in Experimental Procedures. Representative tracings are shown. Identical results were observed in all of five separate experiments.

Fig. 4 Contribution of Ca^{2+} channels to ET-1-evoked increase in $[\text{Ca}^{2+}]_i$. The transfected CHO cells loaded with fura-2 were stimulated with 10^{-8} M ET-1 (A) or 10^{-11} M ET-1 (B) in the absence ① and presence of pretreatment with 10^{-7} M nicardipine ③ or with 4 mM EGTA ②. Panels C and D indicate the effect of the L-type Ca^{2+} channel agonist Bay K 8644 (10^{-7} M) and 20 mM KCl on the increase in $[\text{Ca}^{2+}]_i$ in the transfected cells, respectively. The same results were obtained in all of five separate experiments.

biphasic increase in $[\text{Ca}^{2+}]_i$ as shown in Fig. 3A, whereas at low concentrations (10^{-12} and 10^{-11} M) it caused only a sustained, small but significant increase in $[\text{Ca}^{2+}]_i$ (Fig. 3B).

To investigate the effect of extracellular Ca^{2+} on the ET-induced Ca^{2+} mobilization, the cells were incubated with 4 mM ethylene glycol bis(b-aminoethyl ether)- N,N,N',N' -tetraacetic acid (EGTA) prior to addition of ET. This treatment attenuated the initial, transient phase of the $[\text{Ca}^{2+}]_i$ response induced by 10^{-8} M ET-1 and completely abolished the sustained phase (Fig. 4A, compare ② to ①). A similar result was obtained when cells were pretreated with 0.5 mM NiCl_2 , an inorganic Ca^{2+} channel blocker, indicating that the dependence on extracellular Ca^{2+} reflects influx through Ca^{2+} channels (data not shown). To provide additional characterization of the Ca^{2+} channels involved in this system, the effect of nicardipine, an antagonist of voltage-dependent, L-type Ca^{2+} channels, was examined. Preincubation with 10^{-7} M nicardipine resulted in attenuation of both the transient and sustained phase of the $[\text{Ca}^{2+}]_i$ response evoked by 10^{-8} M ET-1 (Fig. 4A, compare ③ to ①). The sustained $[\text{Ca}^{2+}]_i$ response induced by a low concentra-

tion of ET-1 (10^{-11} M) was also attenuated by the addition of 10^{-7} M nicardipine (Fig. 4B, compare ③ to ①) and completely blocked by 4 mM EGTA (Fig. 4B, compare ② to ①). Nicardipine at 10^{-6} M almost completely prevented the sustained increase in $[\text{Ca}^{2+}]_i$ in response to both high (10^{-10} M $\leq$) and low ($\leq 10^{-11}$ M) concentrations of ET-1 (data not shown). The presence of L-type Ca^{2+} channels was demonstrated by a slow, sustained increase in $[\text{Ca}^{2+}]_i$ in response to 10^{-7} M Bay K 8644, an L-type Ca^{2+} channel agonist (Fig. 4C) and to 20 mM KCl (Fig. 4D). These results suggested that the sustained increase in $[\text{Ca}^{2+}]_i$ occurred mainly through L-type Ca^{2+} channels although the partial involvement of other Ca^{2+} channels such as receptor-gated Ca^{2+} channels cannot be ruled out. The same effects of EGTA, NiCl_2 , or nicardipine were observed in response to ET-3 (data not shown).

Relation between ET-Induced InsP_3 Production and $[\text{Ca}^{2+}]_i$ Increase

Both the ET_A and ET_B receptors have been shown to couple with PLC, which activates the inositol phosphate/ Ca^{2+} second messenger pathways (1, 2,

Fig. 5 Dose-response curves for InsP₃ production and increase in [Ca²⁺]_i in response to ET-1. A: The transfected CHO cells were incubated with indicated concentrations of ET-1 for 1 min, then resultant InsP₃ production was measured. The InsP₃ production was expressed as the fold increase of InsP₃ levels in cells untreated with ET-1. The data represent the mean $\pm$ SE obtained from three separate experiments, each performed in duplicate. B: The transfected CHO cells loaded with fura-2 were stimulated with the indicated concentrations of ET-1 in the presence of 4 mM EGTA. Each point was normalized to the size of the increase in [Ca²⁺]_i induced by 10⁻⁷ M ET-1. The data represent the mean $\pm$ SE obtained from eight experiments.

11, 19). We therefore investigated the production of InsP₃ in response to various concentrations of ET-1 in the permanently transfected CHO cells. As shown in Fig. 5A, InsP₃ production induced by ET-1 (10⁻¹⁰–10⁻⁶ M) increased in a dose-dependent manner with an EC₅₀ value of 4.5×10^{-9} M, suggesting that following binding of ET-1, the ET_B receptor activates PLC, resulting in the formation of InsP₃. On the other hand, ET-1 at 10⁻¹¹ M did not induce InsP₃.

A dose-response curve for the ET-1-induced transient increase in [Ca²⁺]_i with an EC₅₀ value of 4.5×10^{-10} M was obtained under a condition in which extracellular Ca²⁺ was depleted by adding EGTA to the reaction buffer (Fig. 5B). This value was 10-fold lower than that of the ET-1-caused InsP₃ production. It is not clear why these different EC₅₀ values were obtained although this condition should reflect InsP₃-mediated [Ca²⁺]_i increase. One possible explanation is as follows: The existence of a large amount of the recombinant ET_B receptors resulted in a high production of InsP₃. Thus, InsP₃ receptors might be saturated by their ligands and exhibit the maximal [Ca²⁺]_i increase before ET-1-caused production of InsP₃ reaches a maximal level. However, it should be noted that ET-1 at 10⁻¹¹ M,

at concentration where ET-1 had no activity to produce InsP₃, did not increase [Ca²⁺]_i as well. Together with the data from Figs. 3–5 suggested that the ET-stimulated transient increase in [Ca²⁺]_i results from both release of intracellular Ca²⁺ stores mediated by InsP₃ and extracellular Ca²⁺ influx via L-type Ca²⁺ channels, whereas the sustained phase results only from extracellular influx. Therefore, it seems likely that ET at low concentrations ($\leq 10^{-11}$ M) couples with the extracellular Ca²⁺ influx but not with InsP₃ production, leading to release of Ca²⁺ from intracellular stores, whereas ET at high concentrations (10^{-10} M $\leq$) can link to both these systems.

Internalization of ET-ET_B Receptor Complex

It has become increasingly evident that receptors coupled to G proteins such as angiotensin II and muscarinic acetylcholine receptors are internalized, like epidermal growth factor (EGF) and low density lipoprotein (LDL) receptors that possess a single transmembrane domain (4, 5, 10, 15, 27, 29). Resink *et al.* (17) have shown that ET-ET_A receptor complexes are rapidly internalized in cultured vascular smooth muscle cells. To investigate whether

Fig. 6 Internalization of ET-ET_B receptor complex. A: The transfected CHO cells were incubated with 100 pM ¹²⁵I-ET-1 at 37°C for the indicated periods of time, then covalently cross-linked using DSS at 4°C. B: The cells were reacted with 100 pM ¹²⁵I-ET-1 at 4°C instead of 37°C before cross-linking. C: The cells were treated with 500 μM dansylcadaverin for 1 h at 37°C before incubation with 100 pM ¹²⁵I-ET-1 at 37°C. D: The transfected CHO cells previously equilibrated with 100 pM ¹²⁵I-ET-1 for 2 h at 4°C were incubated at 37°C for the indicated periods of time before cross-linking. ¹²⁵I-ET-1-labeled ET_B receptor was analyzed using SDS-PAGE followed by autoradiography. The specific band of 58 kDa for ET-1 is shown.

ET-ET_B receptor complexes are also internalized, we followed the fate of the complexes by affinity labeling. The transfected cells were incubated with 100 pM ¹²⁵I-ET-1 at 37°C for 0.5, 1, 2, and 3 h, covalently cross-linked with the radioligand via DSS, and separated by SDS-PAGE followed by autoradiography. Under these conditions, the binding increased rapidly with time, reached a maximum

after 1 h of association, and then decreased slightly in a time-dependent manner (data not shown). In contrast, as shown in Fig. 6A, the intensity of the affinity-labeled band at 58 kDa declined markedly in a time-dependent manner; after 2 h of incubation, the intensity was less than 10% of that of the maximum. These results demonstrate the possibility that ET-ET_B receptor complexes are internalized at 37°C. Since internalization is inhibited at low temperatures in general, cells were affinity-labeled after incubation with ¹²⁵I-ET-1 at 4°C instead of 37°C. As shown in Fig. 6B, the intensity of the band reached a maximum after 1 h of incubation and remained stable for at least 3 h in contrast to the incubation at 37°C. To further confirm the internalization, cells had been treated with 500 μM dansylcadaverin for 1 h at 37°C before they were reacted with ¹²⁵I-ET-1 at 37°C. Dansylcadaverin is a potent inhibitor of transglutaminase whose activity is necessary for both receptor clustering and the endocytosis process; in CHO cells, 20 μM dansylcadaverin produces a 50% inhibition of α₂-macroglobulin clustering (15). Fig. 6C shows that there was no noticeable change in the appearance of the band after the binding reached a plateau phase as in the incubation at 4°C. Together, these data suggest that following ET-1 binding to the ET_B receptor on the cell surface, the ligand is internalized via receptor-mediated endocytosis similarly as ET_A, EGF, and LDL receptors. The kinetics of internalization at 37°C was analyzed in cells previously equilibrated with ¹²⁵I-ET-1 for 2 h at 4°C to permit binding in the absence of internalization. After unbound radioligands were washed, cells were incubated for 10, 20, and 45 min at 37°C followed by affinity labeling using DSS. In Fig. 6D, the band intensity was rapidly decreased and was almost undetectable after incubation for 45 min; the half time of internalization (*t*_{1/2}) was approximately 15 min. This *t*_{1/2} value was identical to that of ET-ET_A receptor internalization in cultured smooth muscle cells (17).

These findings raised the possibility that equilibrium binding studies performed at 37°C do not accurately reflect the number of ET binding sites on the surface of the transfected cells. Therefore, binding parameters were obtained again by incubating the cells with ¹²⁵I-ET-1 or -3 at 4°C, a temperature at which internalization of ET-receptor complexes does not occur. Fig. 1C shows a typical saturation isotherm for ¹²⁵I-ET-1 binding to the transfected cells. Scatchard analyses of this binding data produced a linear plot, suggesting the presence

of a single population of binding sites (Fig. 1D). The binding parameters determined in three different experiments were $K_d = 77 \pm 22$ pM and $B_{\max} = 1.25 \pm 0.37 \times 10^6$ sites/cell. Similar binding parameters, $K_d = 120 \pm 41$ pM ($n = 3$) and $B_{\max} = 1.17 \pm 0.52 \times 10^6$ sites/cell ($n = 3$), were obtained for ^{125}I -ET-3. The $B_{\max}$ values were approximately 85% of those calculated from equilibrium binding studies performed at 37°C for both ET-1 and -3, indicating that binding studies carried out under conditions where internalization occurs, overestimates the apparent number of surface receptors.

DISCUSSION

One of the major findings of this study is that the ET_B receptor can activate two different signal transduction pathways, depending on the concentrations of ET applied to cells. High concentrations of ET (10^{-10} M $\leq$), at which ET produced the second messenger InsP_3 , caused a rapid, transient increase in $[\text{Ca}^{2+}]_i$ followed by a lower, sustained phase (Fig. 3A). In contrast, low concentrations of ET ($\leq 10^{-11}$ M), at which ET did not induce the production of InsP_3 , caused only a slow, sustained phase of the $[\text{Ca}^{2+}]_i$ response (Fig. 3B). We found that the transient increase in $[\text{Ca}^{2+}]_i$ results from both mobilization of intracellular Ca^{2+} stores and Ca^{2+} influx from outside the cells, whereas the slow, sustained $[\text{Ca}^{2+}]_i$ response completely depends on Ca^{2+} influx. This Ca^{2+} influx was mediated, at least in part, via L-type Ca^{2+} channels. The existence of this channel in the transfected CHO cells is demonstrated by the following findings: (a) the slow, sustained increase in $[\text{Ca}^{2+}]_i$ was induced by the L-type Ca^{2+} channel agonist Bay K 8644 at 10^{-7} M, a concentration at which the reagent is thought to specifically bind to L-type Ca^{2+} channels but not to other ion channels including receptor-gated Ca^{2+} channels; (b) high concentrations of KCl also caused an increase in $[\text{Ca}^{2+}]_i$; and (c) the sustained increase in $[\text{Ca}^{2+}]_i$ induced by both high and low concentrations of ET was significantly attenuated by 10^{-7} M nicardipine, a concentration at which it interacts only with L-type Ca^{2+} channels. We found that expression levels of L-type Ca^{2+} channels in transfected CHO cells differed among isolated clones, but these findings confirm the presence of L-type Ca^{2+} channels in the transfected CHO cells used in the present study. Therefore, although the detailed mechanism of the sustained $[\text{Ca}^{2+}]_i$ response remains unclear, this study demonstrates that ET at low concentrations

($\leq 10^{-11}$ M) couples with Ca^{2+} channel opening, whereas at high concentrations (10^{-10} M $\leq$), it is also linked to activation of PLC despite the existence of only a single receptor type in the transfected cells. Similar phenomena have been reported by Simonson *et al.* (21) using rat cultured mesangial cells; cellular responses to ET change depending on the ligand concentrations with a threshold at about 10^{-11} M. However, mesangial cells contain both types of ET receptors (13). Receptor heterogeneity in the same cells renders understanding the cellular responses to ET complicated. In contrast, our system considerably simplifies investigation of ET actions because only a single type of the receptor exists in the transfected cells. Very recently, Jouneaux *et al.* (7) have reported that the ET_B receptor causes an increase in $[\text{Ca}^{2+}]_i$ via two independent pathways through different types of G proteins in the liver from female rats. The ET_B receptor inhibits the plasma membrane Ca^{2+} pump responsible for Ca^{2+} extrusion out of the cell through a Gs protein. In contrast, the ET_B -induced activation of PLC was mediated by pertussis toxin-insensitive G proteins.

We indicated that after ET binding to the ET_B receptor in the transfected CHO cells, ET- ET_B receptor complexes are rapidly internalized with a $t_{1/2}$ of 15 min via a process termed receptor-mediated endocytosis. Internalization of ligand-receptor complexes is regarded as an important mechanism for disposal of bound ligand and regulation of available receptor sites on the cell surface. However, it has become increasingly evident that internalization is an obligatory step in the generation of target-cell responses. Griendling *et al.* (4) and Hunyady *et al.* (5) have indicated that internalization of AII-receptor complexes is necessary to promote receptor-coupled PLC-mediated phosphoinositide catabolism. Furthermore, Resink *et al.* (17) have revealed that contraction of vascular smooth muscle, which possesses only the ET_A receptor, in response to ET-1 is significantly decreased by dansylcadaverin, a potent inhibitor of receptor clustering and the endocytosis processes. The transfected CHO cells expressing high levels of the ET_B receptor should become a good model to elucidate the physiological roles of internalization of ET- ET_B receptor complexes.

In this study, affinity-labeling was used to follow the fate of ET-receptor complexes during internalization. It is thought that the cross-linking reagent DSS reacts with the ligand-receptor complexes on the cell surface but not with those internalized. In

general, acid treatment is used to differentiate the two binding components, surface-bound and internalized ligands. Acid-sensitive and -resistant binding components are taken to represent surface-bound and internalized ligand, respectively. We could not use acid treatment in our system due to the irreversible binding of ^{125}I -ET-1 or -3 to the ET_B receptor, although this procedure can be used to assess the internalization of ^{125}I -ET-1- ET_A receptor complexes (17). In other words, once bound to the ET_B receptor, the radioligands could not be displaced from the receptor by acid without damaging the cells. Takasuka *et al.* (22) have also reported that ^{125}I -ET-1- ET_B receptor complexes do not dissociate during SDS-PAGE carried out at low temperature, whereas ^{125}I -ET-1- ET_A receptor complexes easily dissociate. This study clearly demonstrates that affinity labeling is a very useful procedure for studying internalization of ligand-receptor complexes as well as acid treatment.

It is of great interest to examine the mechanisms by which the ET_B receptor is linked to two independent signal pathways, depending on the concentrations of ET applied to cells. The biological validity of the internalization should also be important. The transfected cells expressing high levels of recombinant ET_B receptor prepared in this study will become a powerful means in understanding the ET_B receptor-mediated biological functions induced by ET.

We would like to thank Ms Norma Foster for her help in preparing the manuscript. This work was supported by a grant-in-aid from the Ministry of Education, Science and Culture of Japan.

Received 11 July 1994; and accepted 4 August 1994

REFERENCES

1. ARAI H., HORI S., ARAMORI I., OKUBO H. and NAKANISHI S. (1990) Cloning and expression of a cDNA encoding an endothelin receptor. *Nature* **348**, 730-732
2. ARAMORI I. and NAKANISHI S. (1992) Coupling of two endothelin receptor subtypes to differing signal transduction in transfected Chinese hamster ovary cells. *J. Biol. Chem.* **267**, 12468-12474
3. DENUCCI G., THOMAS R., D'ORLEANS-JUSTE P., ANTUNES E., WALDER C., WARNER T. D. and VANE J. R. (1988) Pressor effect of circulating endothelin are limited by its removal in the pulmonary circulation and by the release of prostacyclin and endothelium-derived relaxing factor. *Proc. Natl. Acad. Sci. USA* **85**, 9797-9800
4. GRIENDLING K. K., DELAFONTAINE P., RITTENHOUSE S. E., GIMBRONE M. A. and ALEXANDER R. W. (1987) Correlation of receptor sequestration with sustained diacylglycerol accumulation in angiotensin II-stimulated cultured vascular smooth muscle cells. *J. Biol. Chem.* **262**, 14555-14562
5. HUNYADY L., MERELLI F., BAUKAL A. J., BALLA T. and CATT K. J. (1991) Agonist-induced endocytosis and signal generation in adrenal glomerulosa cells. *J. Biol. Chem.* **266**, 2783-2788
6. INOUE A., YANAGISAWA M., KIMURA S., KASUYA Y., MIYAUCHI T., GOTO K. and MASAKI T. (1989) The human endothelin family: Three structurally and pharmacologically distinct isopeptides predicted by three separate genes. *Proc. Natl. Acad. Sci. USA* **86**, 2863-2867
7. JOUNEAUX C., MALLAT A., GAL C. S.-L., GOLDSMITH P., HANOUNE J. and LOTERSZTAJN S. (1994) Coupling of endothelin B receptors to the calcium pump and phospholipase C via Gs and Gq in rat liver. *J. Biol. Chem.* **269**, 1845-1851
8. KARNE S., JAYAWICKREME C. K. and LERNER M. R. (1993) Cloning and characterization of an endothelin-3 specific receptor (ET_C receptor) from *Xenopus laevis* dermal melanophores. *J. Biol. Chem.* **268**, 19126-19133
9. KOZUKA M., ITO T., HIROSE S., LODHI K. M. and HAGIWARA H. (1991) Purification and characterization of bovine lung endothelin receptor. *J. Biol. Chem.* **266**, 16892-16896
10. LILES W. C., HUNTER D. D., MEIER K. E. and NATHANSON N. M. (1986) Activation of protein kinase C induces rapid internalization and subsequent degradation of muscarinic acetylcholine receptors in neuroblastoma cells. *J. Biol. Chem.* **261**, 5307-5313
11. LIN H. Y., KAJI E. H., WINKEL G. K., IVES H. E. and LODISH H. F. (1991) Cloning and functional expression of a vascular smooth muscle endothelin 1 receptor. *Proc. Natl. Acad. Sci. USA* **88**, 3185-3189
12. MARSAULT R., FEOLDE E. and FRELIN C. (1993) Receptor externalization determines sustained contractile responses to endothelin-1 in the rat aorta. *Amer. J. Physiol.* **264**, C687-C693
13. MARTIN E. R., BRENNER B. M. and BALLERMAN B. J. (1990) Heterogeneity of cell surface endothelin receptors. *J. Biol. Chem.* **265**, 14044-14049
14. MIYAZAKI H., KONDOH M., WATANABE H., MASUDA M., MURAKAMI K., TAKAHASHI M., YANAGISAWA M., KIMURA S., GOTO K. and MASAKI T. (1990) Affinity labeling of endothelin receptor and characterization of solubilized endothelin-endothelin receptor complex. *Eur. J. Biochem.* **187**, 125-129
15. PASTAN I. and WILLINGHAM M. C. (1983) Receptor-mediated endocytosis: coated pits, receptosomes and the Golgi. *Trends. Biochem. Sci.* **8**, 250-254
16. POORMAN R. A., PALERMO D. P., POST L. E., MURAKAMI K., KINNER J. H., SMITH C. W., REARDON I. and HEINRIKSON R. L. (1986) Isolation and characterization of native renin derived from Chinese hamster ovary cells. *Proteins* **1**, 139-145
17. RESINK T. J., SCOTT-BURDEN T., BOULANGER C., WEBER E. and BUHLER F. R. (1990) Internalization of endothelin by cultured human vascular smooth muscle cells. Characterization and physiological significance. *Mol. Pharmacol.* **38**, 244-252
18. SAIDA K., MITSUI Y. and ISHIDA N. (1989) A novel peptide, vasoactive intestinal contractor, of a new (endothelin) peptide family. *J. Biol. Chem.* **264**, 14613-14616
19. SAKURAI T., YANAGISAWA M., TAKUWA Y., MIYAZAKI H., KIMURA S., GOTO K. and MASAKI T. (1990) Cloning of a cDNA encoding a non-isopeptide-selective subtype of the endothelin receptor. *Nature* **348**, 732-735
20. SIMONSON M. S. and DUNN M. J. (1990) Cellular signaling

- by peptides of the endothelin gene family. *FASEB J.* **4**, 2989-3000
21. SIMONSON M. S., WANN S., MENE P., DUBYAK G. R., KESTER M., NAKAZATO Y., SEDOR J. R. and DUNN M. J. (1989) Endothelin stimulates phospholipase C, Na^+/H^+ exchange, c-fos expression, and mitogenesis in rat mesangium cells. *J. Clin. Invest.* **83**, 708-712
22. TAKASUKA T., SAKURAI T., GOTO K., FURUICHI Y. and WATANABE T. (1994) Human endothelin receptor ET_B . *J. Biol. Chem.* **176**, 392-400
23. TAKAYANAGI R., KITAZUMI K., TAKASAKI C., OHNAKA K., AIMOTO S., TASAKA K., OHASHI M. and NAWATA H. (1991) Presence of non-selective type of endothelin receptor on vascular endothelium and its linkage to vasodilation. *FEBS Lett.* **282**, 103-106
24. TAKUWA N., TAKUWA Y., YANAGISAWA M., YAMASHITA K. and MASAKI T. (1989) A novel vasoactive peptide endothelin stimulates mitogenesis through inositol lipid turnover in Swiss 3T3 fibroblast. *J. Biol. Chem.* **264**, 7856-7861
25. TAKUWA Y., KASUYA Y., TAKUWA N., KUDO M., YANAGISAWA M., GOTO K., MASAKI T. and YAMASHITA K. (1990) Endothelin receptor is coupled to phospholipase C via a pertussis toxin-insensitive guanine nucleotide binding regulatory protein in vascular smooth muscle cells. *J. Clin. Invest.* **85**, 653-658
26. TAKUWA Y., TAKUWA N. and RASMUSSEN H. (1986) Carbachol induces a rapid and sustained hydrolysis of polyphosphoinositides in bovine tracheal smooth muscle. *J. Biol. Chem.* **261**, 14670-14675
27. ULLIAN M. E. and LINAS S. L. (1989) Role of receptor cycling in the regulation of angiotensin II surface receptor number and angiotensin II uptake in rat vascular smooth muscle cells. *J. Clin. Invest.* **84**, 840-846
28. WATANABE H., MIYAZAKI H., KONDOH M., MASUDA Y., KIMURA S., YANAGISAWA M., MASAKI T. and MURAKAMI K. (1989) Two distinct types of endothelin receptors are present on chick cardiac membranes. *Biochem. Biophys. Res. Commun.* **161**, 1252-1259
29. WILEMAN T., HARDING C. and STAHL P. (1985) Receptor-mediated endocytosis. *Biochem. J.* **232**, 1-14
30. WILKES B. M., RUSTON A. S., MENTO P., GIRARDI E., HART D., MOLEN M. V., BARNETT R. and NORD E. P. (1991) Characterization of endothelin 1 receptor and signal transduction mechanisms in rat medullary interstitial cells. *Amer. J. Physiol.* **260**, F579-F589
31. XUAN Y. T., WATKINS W. D. and WHORTON A. R. (1991) Regulation of endothelin-mediated calcium mobilization in vascular smooth muscle cells by isoproterenol. *Amer. J. Physiol.* **260**, C492-C502
32. YANAGISAWA M., KURIHARA H., KIMURA S., TOMOBE Y., KOBAYASHI M., MITSUI Y., YAZAKI Y., GOTO K. and MASAKI T. (1988) A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature* **332**, 411-415