Vol. 217, No. 1, 1995 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
December 5, 1995 Pages 354-362

TRUNCATION OF THE RECEPTOR CARBOXYL TERMINUS IMPAIRS MEMBRANE SIGNALING BUT NOT LIGAND BINDING OF HUMAN ET_B ENDOTHELIN RECEPTOR

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Received October 27, 1995

SUMMARY: Human ET_B endothelin receptor (hET_BR) is a heptahelical G-protein-coupled receptor consisting of 442 amino acids whose carboxyl (C) -intracellular region has four and twelve sites for potential palmitoylation and phosphorylation, respectively. In order to elucidate the functional roles of these modification sites, we constructed a series of C-terminal truncated hET_BRs and expressed them in L*tk*$^-$ cells. All the truncated hET_BRs showed ligand-binding profiles similar to those of the wild-type hET_BR. The truncated receptors holding Cys-402 retained both normal intracellular calcium ($[Ca^{2+}]_i$) response and its rapid desensitization; however, the deleted receptors lacking Cys-402 failed to induce the $[Ca^{2+}]_i$ response. These results showed that Cys-402 of hET_BR is necessary for its intracellular calcium signaling and that at least ten of twelve putative phosphorylation sites are irresponsible for the agonist-induced desensitization.

The endothelins are a family of potent vasoactive peptides termed endothelin-1, -2 and -3 (ET-1, -2 and -3) (1, 2). They have a wide variety of biological effects in many different target cell types (3, 4, 5), which are mediated by specific cell surface receptors that belong to the superfamily of heptahelical G-protein coupled receptors (GPCRs). Two distinct subtypes of endothelin receptor, called ET_A and ET_B receptors, have been cloned and characterized (6,7,8). Both receptors activate the phosphoinositides turnover pathway in the target cells, producing a transient elevation of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$). They can be pharmacologically

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Abbreviations: G-protein, guanine-nucleotide-binding-regulatory-protein; Gi, inhibitory G-protein; Gs, stimulatory G-protein; PCR, polymerase-chain-reaction; RT-PCR, reverse transcription-PCR; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

0006-291X/95 $12.00

distinguished by their rank orders of potency towards endothelin isopeptides; ET_A receptor exhibits an affinity rank order of ET-1 ≥ ET-2 >> ET-3, whereas ET_B receptor has similar affinity to all three isopeptides (8).

In general, pharmacological receptors have three characteristics: 1) ligand binding, 2) effector coupling and 3) desensitization. Structural basis for the first two parameters of each human ET receptor have been intensively investigated by mutagenesis studies. For example, it has been demonstrated that the transmembrane domains (TMDs) from IV through VI of human ET_B receptor (hET_BR) and TMDs from I through III and VII of hET_AR are necessary and sufficient for high affinity binding of ET-3 to hET_B and an ET_A-selective antagonist, BQ123, to hET_AR, respectively (9, 10). Selective coupling of intracellular loop (ICL) III of hET_AR and ICLs II and III of hET_BR, respectively, to Gs and Gi, have also been established (11). However, little is known about the molecular basis for desensitization of hETR, although it has been reported that the major carboxyl (C)-terminal region of hET_AR is irrelevant to the agonist-induced desensitization (12).

It is well documented that phosphorylation of serine/threonine residues in the C-terminal intracellular region are essential to the agonist-induced desensitization of the GPCRs (13). Recent studies have also shown that palmitoylation of cysteine residue(s) in the same region is crucial to the membrane signal transduction through the GPCRs (14). The C-terminal tail of hET_BR is quite different from that of hET_AR in its amino acid sequences, while they exhibit high polypeptide sequence similarity to each other within their putative TMDs (≈ 74%) (15, 16). The C-terminal portion of hET_BR contains twelve potential phosphorylation sites, as well as four putative palmitoylation sites. We set out to elucidate the functional roles of the C-terminal tail of hET_B receptor in desensitization and effector coupling of the receptor. We constructed a series of truncated hET_BRs and characterized the mutated receptors expressed in mouse *Ltk*$^-$ cell, in terms of radioligand binding and agonist-induced $[Ca^{2+}]_i$ transient assays.

MATERIALS AND METHODS

Reagents: An ET_B-selective antagonist, RES-701-1 (17) and SRα-based mammalian expression vector pME18Sf$^-$ (9) were kindly provided by Dr. Yuzuru Matsuda (Kyowa Hakko Kogyo Co., Ltd., Tokyo, Japan) and Dr. Kazuo Maruyama (Institute of Medical Science, University of Tokyo, Japan), respectively. Sources of other materials were as follows: ET-1 and ET-3 (Peptide Institute Inc., Osaka, Japan); [^{125}I] ET-1 (specific activity 2,000 Ci/mmol, Amersham Japan Corp., Tokyo, Japan); Erase-a-Base System (Promega Corp., Madison, WI); fetal calf serum, G418 (Geneticin), and Lipofectamine (GIBCO/BRL, Gaithersburg, MD); fura-2/AM (Dojindo, Kumamoto, Japan); platelet-activating factor (PAF: 1-*O*-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine) (Funakoshi Co., Ltd., Tokyo, Japan); Stop-codon linker Nhe-I and ISOGEN reagents (Nippon Gene Co., Ltd., Tokyo, Japan); pBluescript SK (-) (Stratagene, La Jolla, CA); All other chemicals were of reagent grade and were obtained commercially.

Construction of truncated hET_B receptor cDNAs: The cDNA of the wild-type hET_BR was subcloned into pBluescript SK (-). A series of six deleted hET_BRs were generated by using a Erase-a-Base system. A stop-codon linker Nhe-I (5'-CTAACTAATTAGCTAGCTAATTAGTTAG-3') was then inserted into the deleted 3' terminus of hET_B cDNA. As a part of stop-codon linker sequence, several extra amino acids were incorporated just before the stop codon of five mutant receptors (Fig. 1).

Other truncated hET_BR cDNAs ($hET_B\Delta40$, $hET_B\Delta41$ and $hET_B\Delta43$) were created by PCR. The nucleotide sequences of oligonucleotide primers were as follows:
UP1, 5'-TTCCCCTTCACCTCAGCAGGATTC-3' (5'-primer);
LW1, 5'-CCAGCAGCATAA**TCA**TGACTTAAAG-3' (3'-primer);
LW2, 5'-CCAGCA**TCA**TAAGCATGACTTAAAG-3' (3'-primer) and
LW3, 5'-CCA**TCA**GCATAAGCATGACTTAAAG-3' (3'-primer).
Each 3'-primer, LW1, LW2 or LW3, contains one nucleotide substitution (as indicated by bold face) to introduce a termination stop codon (as indicated by underline) at codon 400, 402 and 403, respectively (Fig. 1). The 203 bp fragments were amplified by 5'-primer UP1 and each 3'-primer from hET_BR cDNA as a template. The PCR amplification profiles were: denaturation at 94 °C for 1 min, primer annealing at 55 °C for 30 sec and extension at 72 °C for 1 min, for 30 cycles. The PCR products were subcloned into the pBluescript SK (-), then cut out with EcoRI and XbaI, and inserted the EcoRI/XbaI site of $pBSK/hET_B$ to substitute 3'-terminal sequence of the wild-type receptor. The cDNAs of the wild-type or mutant hET_BRs were then transferred into the SRα promoter-based mammalian expression vector pME18Sf⁻ (9) and the nucleotide sequences were confirmed by 373A DNA autosequencer (Applied Biosystems, Inc., Foster City, CA).

Generation of stable transfectants with truncated hET_B receptors: Cell culture and transfection were performed as described previously (11, 18). Briefly, *Ltk*⁻ cells, grown in 5% CO_2 at 37 °C, were plated at a density of $5x10^6$ cells/10-cm plate in 6 ml of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, and allowed to attach overnight. Ten μg of wild-type or truncated hET_BR constructs were co-transfected with 1 μg of pSV2neo plasmid using polycationic lipid Lipofectamine. Clonal cell lines resistant to Geneticin (0.5 mg/ml) were selected for further screening by RT-PCR. Total RNA prepared by ISOGEN reagents was reverse transcribed and subjected to PCR. The primer set of
5'-TCTCTGTGGTTCTGGCTGTC-3' (5'-primer) and
5'-TGCTGAGGTGAAGGGGAAGC-3' (3'-primer)
were used to amplify a 345 bp DNA fragment of hET_BR, with a 30 cycle in the following condition; 94 °C for 1 min, 55 °C for 30 sec and 72 °C for 1 min. The PCR products were subjected to the PAGE and visualized with ethidium bromide.

Low Temperature (LT) SDS-PAGE Analysis: LT SDS-PAGE analysis was performed as described previously (19). Briefly, each stable transfectants plated into 35-mm dish were incubated in 250 ml of buffer A (DMEM containing 30 mM HEPES (*N*-[2-Hydroxyethyl]piperazine-*N*'-[2-ethanesulfonic acid]) and 0.1% bovine serum albumin, pH 7.4) containing 100 pM [^{125}I]ET-1 at 4 °C for 4 h, washed five times with ice-cold buffer A, and lysed with 100 μl of sample buffer (0.25 M Tris-HCl, 2% SDS, 20% glycerol and 0.01% bromophenol blue, pH 6.8). The lysates were then applied to 10% acrylamide-0.1% SDS gels at 20 μg protein per lane. The electrophoresis was performed at a constant current of 40 mA with keeping the plate at 4 °C. The gels were then dried at 85 °C for 1 hr and autoradiographed by using BAS 2000 (Fuji, Tokyo, Japan). The protein concentration was measured using the BCA microprotein assay kit (PIERCE, Rockford, IL, USA).

[^{125}I]ET-1 Binding Assay: [^{125}I]ET-1 binding assays were performed as described previously (11). For saturation binding studies, increasing concentrations (10 - 3,000 pM) of [^{125}I]ET-1 were incubated with crude membrane preparations at 37 °C for 60 min. For competition binding assays, the membrane preparations were incubated at 37 °C for 60 min with 30 pM of [^{125}I]ET-1 and various concentrations of ET-1, ET-3 and RES-701-1. Non-specific binding was defined in the presence of 300 nM unlabeled ET-1 and was always less than 10% of the total binding.

Measurements of Intracellular Ca^{2+} Transients: Intracellular Ca^{2+} transients evoked by various concentrations of ET-1 were monitored by a JASCO CAF-110 fluorescence spectrophotometer as described previously (18). ET-1 induced an acute $[Ca^{2+}]_i$ increase that was followed by lower plateau $[Ca^{2+}]_i$ levels. The peak $[Ca^{2+}]_i$ values from the initial transients were used to generate the dose-response curves. In some experiments, 10 μM RES-701-1 was added 5 min prior to the addition of ET-1.

Data analysis: All data were the means of at least three independent experiments done in duplicate. Raw data obtained from the radioligand binding and Ca^{2+} transient assays were fitted to logistic equation by using a non-linear least-squares curve-fitting program (9).

RESULTS

Characterization of C-terminal truncated hET_B receptors: Nine truncated hET_BRs were constructed by progressive removal of the C-terminal amino acid residues. We designated truncated hET_BRs as $hET_B\Delta X$, where X represents the number of truncated amino acids. The truncated hET_BRs we constructed were $hET_B\Delta 11$, $\Delta 23$, $\Delta 37$, $\Delta 39$, $\Delta 40$, $\Delta 41$, $\Delta 43$, $\Delta 46$, $\Delta 47$ (Fig 1). Each deletion molecules was stably expressed in mouse L*tk*$^-$ cells and at least three clonal cell lines were subjected to RT-PCR assay. Expression of hET_BR transcript was confirmed by amplification of a 345 bp DNA fragment corresponding to 683-1,027 nucleotides of hET_BR cDNA (Fig 2).

We further ascertained that each truncated hET_BR protein did exist in the stable transfectant by LT SDS-PAGE assay. As previously reported, LT SDS-PAGE analysis with wild-type hET_B receptor showed a broad 50 kDa and a sharp 35 kDa bands, corresponding the full length and the N-terminal proteolytic hET_BR, respectively (20)(Fig. 3, lane 2). Excess application of cold ET-1 (100 nM, lane 1) or an ET_B selective antagonist, RES-701-1 (100 μM, data not shown), abolished these bands, showing the specific binding of [^{125}I]ET-1 to these hET_BRs and their proteolytic products. Expected molecular weight bands, which were smaller than that of wild type hET_BR as estimated from the truncated receptor cDNAs, were indeed revealed (Fig. 3).

Finally, we chose stable transfectants expressing similar densities of [^{125}I]ET-1 binding sites in these cells; maximal specific binding sites (*Bmax*) of [^{125}I]ET-1 were between 477 and 2,752 pmol per mg protein. Table 1 summarizes the *Bmax* and apparent *Ki* values of the competitors determined for the wild-type and truncated hET_BRs expressed in L*tk*$^-$ cells. The *Ki* value for ET-1 was similar in the wild-type and all deleted Rs, all being within the range from 70 to 190 pM. The *Ki* values for ET-3 or RES-701-1 determined in this study were also similar to those of wild-type hET_BR, being within the range from 34 to 83 pM, from 12 to 36 nM, respectively.

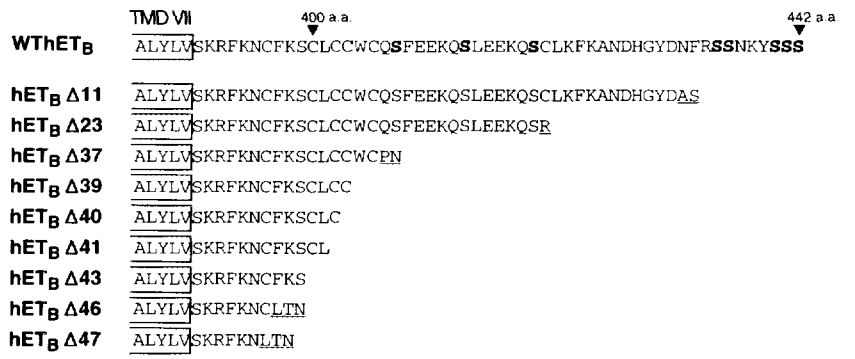

Fig. 1. C-terminal amino acid sequences of wild-type and truncated hET_B receptors. C-terminal 58 amino acid sequences (from residue 385 to 442) of wild-type hET_BR (WThET_B) are shown on the top, and truncated hET_BRs below. The solid box outlines the possible sites for palmitoylation. All serine residues distal to Cys-402 are depicted in the bold face. Underlined are extra amino acids (artificially) added to the deleted C-terminal ends. TMD VII refers to the C-terminal portion of the putative seventh transmembrane-spanning domain.

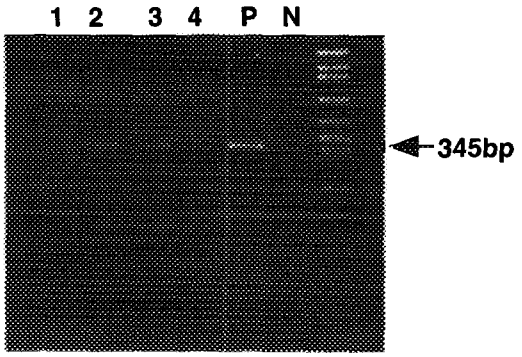

Fig. 2. RT-PCR analysis of the expression of mutated hET_B receptor transcripts in geneticin-resistant clonal cell lines. 345-bp DNA fragments were amplified by PCR. The nucleotide sequences of the primers and the reaction conditions of PCR were described in detail under Materials and Methods. lane 1, parental L*tk*$^-$ cells; lane 2, hET_BR; lane 3, $hET_B\Delta46$ receptor; lane 4, L*tk*$^-$ cells transfected with mock cDNA. P and N indicate positive (lane 5) and negative (lane 6) controls, respectively.

Intracellular Ca^{2+} Transient Response: ET-1 (100 nM) caused a rapid elevation of $[Ca^{2+}]_i$ that was followed by lower plateau $[Ca^{2+}]_i$ levels, through wild-type hET_BR (Fig. 4A). L cells expressing the mutated hET_BRs that were deleted up to 40 amino acid residues of the original C-terminus ($ET_B\Delta11$, $\Delta23$, $\Delta37$, $\Delta39$, $\Delta40$) exhibited almost similar $[Ca^{2+}]_i$ responses to that of wild-type hET_BR by ET-1 (100 nM). These ET-1 (100 nM)-induced $[Ca^{2+}]_i$ responses were completely suppressed by pretreatment with RES-701-1 (10 μM). Fig. 4B shows the representative $[Ca^{2+}]_i$ response mediated by $ET_B\Delta40$: the peak $[Ca^{2+}]_i$ value was 1110 ± 190 nM (n=4), being similar to 1270 ± 270 nM (n=4) of wild-type hET_BR (Fig. 4A, 4B). The ET-1 concentrations that elicited half-maximum response (EC_{50}) for these wild-type and truncated hET_BRs were all within a range between 0.8 and 2.1 nM (Table 1). In contrast, ET-1 failed to

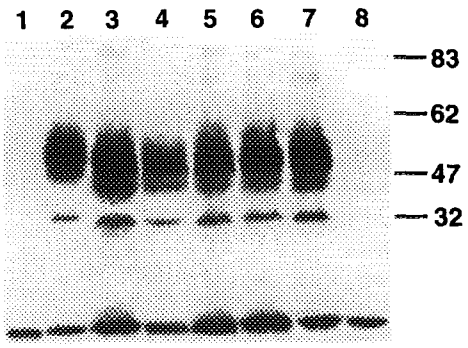

Fig. 3. Detection of the complexes of [^{125}I]ET-1 with wild-type or mutated hET_B receptors by LT SDS-PAGE analysis. L cells expressing wild-type or mutated hET_BRs were incubated with 100 pM [^{125}I]ET-1 for 4 hrs at 4 °C. The whole cell homogenates were subjected to SDS-PAGE under low temperature condition (4 °C). lane 1, wild-type hET_BR with excess amount of cold ET-1 (100 nM). lane 2, wild-type hET_BR. lanes 3-7, the truncated hET_BRs; $hET_B\Delta39$, $\Delta40$, $\Delta41$, $\Delta43$ and $\Delta47$ cells. lane 8, mock transfection (vector alone). Given on the right are the sizes (kDa) of the molecular weight markers. Note that each band expected for $hET_B\Delta39$, $\Delta40$, $\Delta41$, $\Delta43$ and $\Delta47$ cells (lanes 3-7) showed smaller size than that for wild-type hET_B by approximately 4.6, 4.7, 4.8, 5.0 and 5.2 kDa, respectively.

Table 1. Summary of binding and functional properties of wild-type and mutated hET_B receptors stably expressed in L cells

	Binding Studies				$[Ca^{2+}]_i$ responses	
	Bmax (pmol/mg protein)	*Ki* (pM)	(pM)	(nM)	EC_{50} (nM)	$\Delta[Ca^{2+}]_i$ (nM)
	ET-1	ET-1	ET-3	RES-701-1	ET-1	PAF 1 μM
$WThET_B$	596	77	34	21	1.5	1,180
$hET_B\Delta11$	998	180	42	16	1.6	1,220
$hET_B\Delta23$	784	165	n.d.	n.d.	1.2	1,290
$hET_B\Delta37$	1004	130	n.d.	n.d.	1.8	1,150
$hET_B\Delta39$	483	190	n.d.	n.d.	0.8	1,340
$hET_B\Delta40$	845	190	66	53	2.1	1,220
$hET_B\Delta41$	670	76	49	19	N.R.	1,280
$hET_B\Delta43$	477	106	n.d.	n.d.	N.R.	1,170
$hET_B\Delta46$	1055	90	83	12	N.R.	1,270
$hET_B\Delta47$	2752	70	61	36	N.R.	1,290

Binding studies were performed with membrane preparations of L cells stably expressing each hET_BR cDNA. *Bmax* was obtained with using [^{125}I]ET-1 as a ligand. For the concentration-response studies, fractional increase in $[Ca^{2+}]_i$ evoked by each concentration of ET-1 was determined as percent of the maximal response induced by 100 nM ET-1, and the effective concentrations (EC_{50}) necessary for inducing the half maximal resoponse were determined. Values are means of at least two separate experiments done in duplicate or triplicate. n.d., not determined; N.R., no response.

elicit $[Ca^{2+}]_i$ response through truncated hET_BRs lacking Cys-402 ($ET_B\Delta41$, $\Delta43$, $\Delta46$, $\Delta47$). In L cell expressing $hET_B\Delta41$, for example, ET-1 (at up to 10^{-6} M) was not able to induce any detectable level of $[Ca^{2+}]_i$ response, whereas PAF (10^{-4} M) produced $[Ca^{2+}]_i$ response through endogenous platelet-activating-factor (PAF) receptor (Fig. 4C).

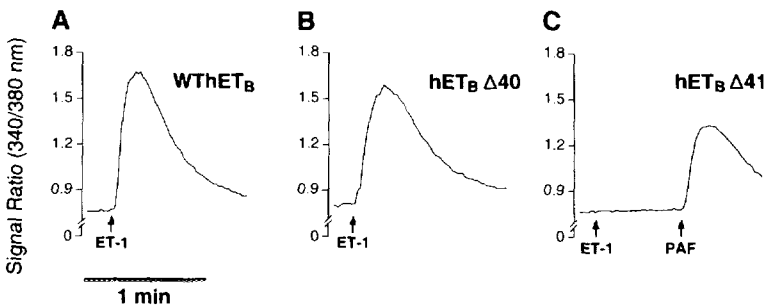

Fig. 4. Effect of the C-terminal deletion of hET_B receptor on the receptor-mediated $[Ca^{2+}]_i$ responses. Typical $[Ca^{2+}]_i$ responses induced by ET-1 (100 nM) in L cells expressing wild-type hET_B (A), $hET_B\Delta40$ (B) and $hET_B\Delta41$ (C) are shown. At least four independent experiments were carried out for each cell type. The peak responses, as expressed in Ca^{2+} concentration, were 1270 ± 270 nM (n=4) and 1110 ± 190 nM (n=4) for wild-type hET_B and $hET_B\Delta40$, respectively. Note that, in L cells expressing $hET_B\Delta41$, ET-1 (100 nM) failed to induce the $[Ca^{2+}]_i$ response, while, on the other hand, PAF (1 μM) elicited the response of reasonable magnitude.

Agonist-Induced Desensitization of Truncated hET_B Receptors: We adopted the following experimental protocol to examine the agonist-induced rapid desensitization of wild-type and the "active" truncated hET_BRs ($hET_B\Delta11$, $\Delta23$, $\Delta37$, $\Delta39$, $\Delta40$). L cells expressing wild-type or mutated hET_BRs were sequentially exposed to ET-1 at lower and higher doses; the first exposure to ET-1 at 0.5 nM (significantly lower than EC_{50}) for 5 min was followed by the second application of ET-1 at 100 nM (supra-maximal concentration). In L cell expressing wild-type hET_BR, significant transient $[Ca^{2+}]_i$ increase (321 ± 45 nM, n = 4) was observed at the first application of ET-1 (0.5 nM). This 5 minute pretreatment abolished the expected $[Ca^{2+}]_i$ response to the second challenge of ET-1 (100 nM), while the cell still held the capability to increase $[Ca^{2+}]_i$ by PAF (1 μM) (Fig. 5A, the upper panel). All the "active" truncated hET_BRs developed the rapid desensitization induced by ET-1, quite similarly to wild-type hET_BR. ET-1 (0.5 nM) elicited the transient $[Ca^{2+}]_i$ response (330 ± 31 nM, n = 3) but the second application of ET-1 failed to induce detectable increase in $[Ca^{2+}]_i$, even in cells expressing the mutated

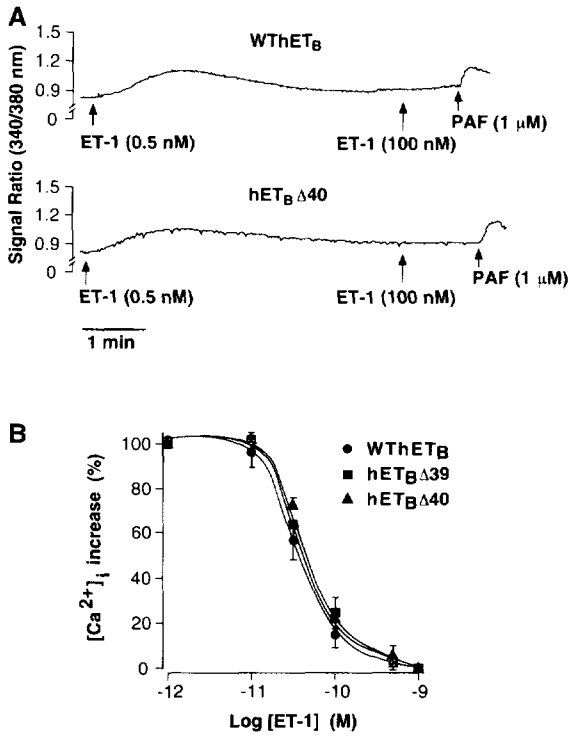

Fig. 5. Effect of the C-terminal deletion of hET_B receptor on the ET-1-induced-homologous desensitizaion. (A) Representative $[Ca^{2+}]_i$ responses induced by sequential application of ET-1 in L cells expressing wild-type hET_B (top) and $hET_B\Delta40$ (bottom). Six separate experiments were performed for each cell type. Note that preincubation of ET-1 (0.5 nM) abolished the second response to ET-1(100 nM), while the following application of PAF (1 μM) increased $[Ca^{2+}]_i$. (B) Concentration-dependent development of the ET-1-induced desensitization of wild-type hET_B, $hET_B\Delta39$ and $hET_B\Delta40$ receptors. Illustrated are the relationships between the concentrations of the first ET-1 (applied for 5 minutes) and the fractional $[Ca^{2+}]_i$ responses by the second ET-1 (100 nM), expressed in percent by regarding the following response by PAF (1 μM) as 100 %. Each point represents the mean ± S.E.M. of at least three separate experiments done in duplicate.

hET_BR with the largest deletion, $hET_B\Delta40$ receptor lacking eight serine residues (Fig. 5A., the lower panel). The development of this rapid desensitization of hET_BRs was concentration-dependent. The half-maximal concentrations of ET-1 to inhibit the second response (IC_{50}) was 24 ± 5 pM for wild-type hET_BR. The IC_{50} values for $hET_B\Delta39$ and $hET_B\Delta40$ receptors were almost identical to that of wild-type hET_BR: 29 ± 7 pM, n = 4, and 28 ± 8 pM, n = 4, respectively (Fig. 5B).

DISCUSSION

We ascertained that the truncated hET_BR proteins corresponding to each mutated cDNA were indeed expressed in *Ltk*$^-$ cells, by using LT SDS-PAGE with [^{125}I] ET-1. L cells stably expressing similar densities of specific [^{125}I]ET-1 binding sites were utilized for the further studies. All the deleted receptors exhibited high affinities to ET-1, ET-3 and RES-701-1 that were comparable with those observed in the wild-type ET_BR. These findings suggest that the details of tertiary structure of the hET_BR are well maintained in all the truncated hET_BRs tested.

By systematically constructing and analysing truncated hET_BRs exhibiting these prerequisite properties, we demonstrated that a single cysteine residue in the C-terminal tail of hET_BR was essential to mediate intracellular calcium signaling, and that the agonist-induced rapid desensitization of hET_BR is less likely developed by phospholyration of its C-terminal stalk. Functional requirement of corresponding cysteine residue has also been reported for human β_2-adrenergic receptor (21). It is of interest to examine whether the crucial role of these conserved cysteine residues can also be seen in the membrane signaling through other family of heptahelical G-protein coupled receptors (12, 22, 23, 24).

We have demonstrated that Cys-402 in hET_BR is essential to its intracellular calcium response. Possible requirement of the corresponding cysteine residue in hET_AR to elicit inward chloride current has previously been shown in *Xenopus laevi* oocytes (12). Cys-402 is conserved in all the G-protein coupled receptor superfamily and is considered as a potential palmitoylation site (13). The putative palmitoylation may be required for selective coupling to a subclass and/or basic recognition of G-proteins (14). We have also shown that the major portion of the C-terminal tail of hET_BR, including ten out of twelve putative phosphorylation sites (25), were not involved in the agonist-induced rapid desensitization. The delineation of responsible structures of hET_BR for this homologous desensitization awaits for further studies .

It has been recently reported that W276C point mutation of hET_BR resulted in partial impairment of $[Ca^{2+}]_i$ increase and caused Hirschsprung's disease in one Mennonite kindred (26). In this study, we created truncated hET_BRs which completely lack the ability to induce the $[Ca^{2+}]_i$ response. These mutated hET_BR cDNAs ($hET_B\Delta41$, $\Delta43$) were created by a single base substitution (C to A) in cysteine codon (TG<u>C</u>), resulting in termination codon (TG<u>A</u>). Thus, natural point mutations in hET_B gene resulting in similar premature termination might cause severe forms of Hirschsprung's disease. The present study would provide the basic structural information regarding the roles of the C-terminal region of the human ET_BR in physiological and pathophysiological settings.

Acknowledgments: We thank Dr. Yuzuru Matsuda of Kyowa Hakko, Co. Ltd. for providing RES-701-1. This work was supported in part by research grants from the Ministry of Education, Science and Culture of Japan (to K. O., G. T. and T. M.) and from Japan Human Science Foundation (to K. O. and G. T.). A. S. is a National Institute Post-Doctral Fellow and thanks the Research Development Corporation of Japan (JRDC) for financial support.

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