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C-terminus of ET_A/ET_B receptors regulate endothelin-1 signal transmission[‡]

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Abstract

The dimerization of the G protein-coupled receptors for endothelin-1 (ET-1), endothelin A receptor (ET_A) and endothelin B receptor (ET_B), is well established. However, the signaling consequences of the homodimerization and heterodimerization of ET_A and ET_B is not well understood. Here, we demonstrate that peptides derived from the C-termini of these receptors regulate the signaling capacity of ET-1. The C-termini of the ET_A and ET_B receptors are believed to consist of three α -helices, which may serve as points of interaction between the receptors. The third α -helix in the C-terminus is of particular interest because of its amphipathic nature. In a cell line expressing only the ET_A receptor, expression of residues Y430–S442, representing the third helix of the ET_B C-terminus, leads to a dramatic increase in the signaling induced by ET-1. In contrast, in a cell line containing only ET_B, Y430–S442 has an antagonistic effect, slightly reducing the ET-1 induced signal. Computational docking results suggest that the α -helical ET_B-derived peptide binds to the second and third intracellular loops of the ET_A receptor consistent with the alteration of its signaling capacity. Our results described here provide important insight into ET_A/ET_B receptor interactions and possibly a new approach to regulate specific G protein-coupled receptor signal transmission.

Keywords

G protein-coupled receptor; endothelin receptors; receptor C-terminus; receptor heterodimerization; ERK signaling

Introduction

Endothelin-1 (ET-1) is a vasoactive peptide signaling through two G protein-coupled receptor (GPCR) isoforms, endothelin A and endothelin B. In the arterial endothelium, the ET_B receptor is the primary isoform expressed [1]. In the smooth muscle of the artery, both receptors are co-expressed [2]. In the endothelium, ET_B acts to produce vasorelaxing factors such as nitric oxide and prostacyclin [3]. In the smooth muscle, the exact function of the ET_B has not been resolved. Both receptors signal via tyrosine/serine kinase cascades such as the RAF/MEK/MAPK pathway [2,4].

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Due to their vasoconstrictive function, the ET_A/ET_B receptors are targets for therapy in vascular diseases. In fact, nonselective, dual receptor blockers such as bosentan are used in the treatment of pulmonary arterial hypertension [5–7]. Although it is clear that ET_A/ET_B antagonists have clinical value in reversing some symptoms of pulmonary arterial hypertension, it is less clear whether blocking the entire signaling actions of both receptors is necessary and most likely not physiologically beneficial to the disease. Additionally, the nonselective antagonists have undesirable side effects such as induction of liver enzyme abnormalities and retention of fluid [8,9].

For a number of years we have been characterizing the roles of the cytoplasmic domains in the signaling of the GPCRs for the vasoactive peptide ligands endothelin, bradykinin, and angiotensin [10–18]. Using receptor chimera and site-directed mutations, we have shown that the cytoplasmic domains of the receptors are highly interactive and that these interactions are very sequence sensitive. For example, mutations/changes in the second intracellular loop (IC2) that suppress signaling can be overcome (signaling restored) by changes in the third intracellular loop or C-terminus. Using the structural features from NMR and molecular modeling we have developed structure–activity relationships for the interactions between the cytoplasmic domains. The possibility of interactions among the cytoplasmic regions is further enhanced by the dimerization, both homodimerization and heterodimerization of the receptors. There is strong evidence that the ET_A and ET_B receptors form dimers [19–21].

Here, we demonstrate that specific segments of the C-termini of the ET_A and ET_B receptors can alter the signaling capacity of the other receptor subtype. These inter-receptor interactions of the cytoplasmic domains may afford a novel target for regulation of ET-1-initiated signaling.

Methods

Homology Modeling

Structural models of the C-termini of the ET_A and ET_B receptors were generated using the program MODELLER [22,23]. The C-terminus of the rat bradykinin-2 (BK₂) receptor, previously characterized by high-resolution NMR [17], was used as a template. The sequence homology of the C-termini of the receptors is high, with a sequence similarity of 53% for ET_A/BK₂ and 61% for ET_B/BK₂.

The homology models for transmembrane helices of the ET_A (EDNRA_human, P25101) and ET_B (EDNRB_human, P24530) receptors were created using SWISS-MODEL [24–26]. The homology models were built using the following templates: the nociceptin receptor (chain B of PDB ID: 4EA3) for ET_A, and the turkey beta1 adrenergic receptor (PDB ID: 4AMJ chain B) for ET_B. The structural features of the C-termini were incorporated into the models by using the hydrophobic clustering or amphipathic nature of the helices as a guide. The first helix (the most N-terminal helix) was placed lying along the membrane surface, perpendicular to the orientation of the seven-transmembrane helices. The next helix (the central helix of the C-terminus) was likewise adjusted to place the hydrophobic amino acids projecting towards the membrane surface, whereas the third helix, highly charged with few hydrophobic residues, was placed randomly before the energy minimization steps.

Energy Minimization

The models of the ET_A and ET_B receptors were subjected to energy minimization and molecular dynamics to relax the homology structures. The NAMD program was used for the simulations and VMD used for viewing and graphic manipulation [27,28]. The energy minimization and MD simulations were carried out in a membrane environment by using the

standard protocol developed for membrane protein simulations provided within NAMD [29]. The total simulation time was 1 ns with 2 fs/step at constant temperature at 300 K. These energy minimized protein structures were used for subsequent docking experiments.

Docking Experiment

Multiple blind docking experiments were carried out for the third helical peptide of ET_B, E3B (Y430–S442; YDNFRSSNKYSSS) against the ET_A and ET_B receptors by using AutoDock 4.0 [30]. The search space (the grid box) for these dockings was limited to the intercellular face of the receptors. After searching with a large grid box encompassing the entire cytoplasmic face, each hit (a promising binding mode of the peptide) was further refined by reducing the grid to this location, thus allowing for finer grain searching. During all of the docking calculations, the peptide was assumed to maintain a helical structure.

Transfection

For this study we chose to concentrate on small peptide segments of the far C-terminal domains of the ET_A and ET_B receptors. Peptide E3A consists of amino acids A415–N427 from the C-terminus of ET_A, and peptide E3B consists of amino acids Y430–S442 from the C-terminus of ET_B. Each peptide was reverse translated into sense and antisense oligonucleotides with an added *Xba*I restriction site followed by a translation initiation sequence upstream of the start codon and *Xho*I restriction site downstream of the stop codon for cloning into the pcMIN zeo DNA expression vector. All pairs of oligonucleotides (i.e. sense and antisense for each C-terminal peptide) were annealed and ligated into pcMIN zeo vector doubly digested with *Xho*I and *Xba*I. Oligonucleotide sequences used in this study are:

E3A:

CTAGAAGCTTACCATGCACAACACAGACCGGAGCAGCCATAAGGACAGCAT
GAACTGAC

cE3A:

TCGAGTCAGTTCATGCTGTCCTTATGGCTGCTCCGGTCTGTGTTGTGCATGGT
AAGCTT

E3B:

CTAGAAGCTTACCATGTATGACAACTTCCGTTCCAGTAATAAATACAGCTCA
TCTTGAC

cE3B:

TCGAGTCAAGATGAGCTGTATTTATTACTGGAACGGAAGTTGTCATACATGG
TAAGCTT.

cDNAs encoding the ET_A or ET_B receptors were stably transfected previously into wild-type Chinese hamster ovary cells by using neomycin as the selecting agent (CHO) [2,31,32]. In this study the CHO-ET_A or CHO-ET_B expressing cells were then stably cotransfected with an additional pcMIN zeo bicistronic vector containing cDNA encoding peptides E3A or E3B and a zeocin resistance gene. CHO-ET_A and CHO-ET_B cotransfected with pcMIN without an E3A or E3B peptide cDNA but containing a zeocin resistance gene were used as controls. To ensure expression of these genes, cells were maintained in the selection medium containing 0.5 mg/ml geneticin (Promega, Madison, WI) and 0.25 mg/ml zeocin (Invitrogen, Carlsbad, CA) during all experiments.

Detection of ERK Activity

Cells were seeded in 6-well cell culture plates until reaching confluence. At that point, they were serum starved for 30 min and then treated with or without 10 nM ET-1 for 5 min. This

time span had previously been found to be optimal in detecting phosphorylated ERK [2,31]. Afterwards, cells were lysed using a 100 μ l RIPA buffer [150 mM NaCl, 1.0% Igepal CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0 (Sigma, St Louis, MO)] and a 1 $\times$ complete protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN), and the total protein was isolated from each well. Total protein was quantified using a bicinchoninic assay. Equal amounts of protein were loaded and electrophoresed on sodium dodecyl sulfate polyacrylamide gel electrophoresis with a 4% stacking and 10% separating gel. Proteins were then transferred to a nitrocellulose membrane (BioRad, Hercules, CA) at 100 V and 4 $^{\circ}$ C for 1 h. The nitrocellulose membranes were blocked at room temperature for 1 h with 5% powdered milk in Tris-buffered saline with Tween 20 (TBS-T) (20 mM Tris, 150 mM NaCl, 0.1% Tween 20, pH 7.6) before incubating with primary rabbit antibody for pERK1/2 (Cell Signaling, Danvers, MA) overnight at 4 $^{\circ}$ C. Diluted 1 : 1000 in a solution TBS-T with 5% bovine serum albumin. The membranes were washed in TBS-T followed by a 1-hour room temperature incubation in the corresponding anti-rabbit secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1 : 10 000 TBS-T. Membranes were washed again in TBS-T and developed in Amersham ECL chemiluminescent detection solution (GE Healthcare) for 1 min before exposure to X-ray film. Using RestoreTM PLUS Western Blot Stripping Buffer (Thermo Scientific, Rockford, IL), the membranes were stripped and likewise probed for glyceraldehyde-3-phosphate dehydrogenase (Cell Signaling, Danvers, MA) as a loading control. Films were scanned with an Epson Perfection 3170 scanner using an Epson Scan (version 1.22A) software. The images were then analyzed using the NIH ImageJ image analysis software to determine the intensity of each band. Relative ERK activity was measured by first normalizing all ERK bands with glyceraldehyde-3-phosphate dehydrogenase and then using that normalized ERK intensity value of E3A or E3B expressing cells divided by ERK band intensity values of empty vector control cells.

Results

The models of the C-termini of the ET_A and ET_B receptors were developed using homology modeling and the structure of the C-terminus of the BK₂ receptor, previously determined by high-resolution NMR [17]. The resulting structures consisting of three α -helices are shown in Figure 1. The first helix (the most N-terminal helix in the peptide) is commonly observed in GPCRs and is often referred to as the eighth helix. For both ET_A and ET_B, this helix is very amphipathic in nature and enriched with cysteines, which serve as possible sites of palmitoylation. In contrast, the second helix, consisting of S397–W407 and E410–K421 for ET_A and ET_B, respectively, has a more random pattern of hydrophobic and hydrophilic amino acids. The last helix of the C-termini of ET_A and ET_B, consisting of Q412–M426 and Y430–S442, respectively, are the longest of the three and consist largely of hydrophilic and charged amino acids.

The homology models of the ET_A and ET_B receptors were generated using standard techniques are shown in Figure 2. During the energy minimization and MD simulations, carried out in a lipid environment, the helices maintain close contact with the membrane, particularly true for the first two helices of the C-termini. The third helix, which was placed randomly, also adopts close interactions with the membrane environment. It is interesting to note that for both receptors, the C-terminus is in close proximity to the IC2. We have previously demonstrated using site directed mutagenesis that with respect to signaling of the BK₂ receptor, there is an interaction between tyrosine residues within the IC2 and C-terminus [10].

To decipher the signaling capacity of each receptor individually, we developed CHO cell lines stably expressing only ET_A or ET_B and, in a separate procedure, also stably expressing

the E3A and E3B C-terminal motifs. We then tested for the ability of these third helical segments (E3A and E3B) to alter ET-1-activated receptor signaling. The results are shown in Figure 3A and B. In CHO-ET_A, the expression of E3A reduced ET-1-induced ERK activity, whereas E3B greatly increased ERK activity compared with cells expressing an empty vector (Figure 3A). This opposing action was also seen in CHO-ET_B where E3A increased ET-1-induced ERK activity and E3B reduced ERK activity compared with cells expressing an empty vector (Figure 3B).

Given the significant alteration of ET_A signaling induced by E3B, we searched for possible modes of binding of this 13-residue long peptide, assumed to be α -helical, to the cytoplasmic face of the receptor. The energy-minimized and MD-relaxed homology models were used for a series of docking simulations that targeted different regions within the cytoplasmic face of the receptors. The lowest energy complex structure for E3B binding to the ET_A receptor is shown in Figure 4. There are several interactions between the E3B peptide and the cytoplasmic face of the ET_A receptor (Figure 5), particularly involving the first and second helical regions of the C-terminus of ET_A. There are hydrogen bonds and Coulombic interactions involving one face of the E3B helix (N432, S436, N437, and S440) with corresponding residues of the C-terminus and IC2 of ET_A. In contrast, there is little interaction of the E3B with the ET_B receptor (Figure 4). Despite extensive docking calculations with various grid search boxes and energy minimization calculations, a similarly stable complex was not observed.

Discussion

We previously demonstrated interplay among the various cytoplasmic domains of the GPCRs for the vasoactive peptide bradykinin and angiotensin [12–17]. Modifications within the intracellular loop that were deleterious to receptor signaling could be overcome with corresponding modifications within the other regions within the cytoplasmic domains [17]. These results in sum demonstrated that the three intracellular loops and C-terminus together function as signaling motifs with partially overlapping and interactive roles.

Recently, we showed that exposure of the ET_B receptor to a cell permeable peptide consisting of the third intracellular loop of the ET_B receptor either increased or decreased ET_A/ET_B signaling [31]. These differential actions by the peptide were puzzling. The C-terminus of the ET_A/ET_B receptors was then chosen for study to obtain a better understanding of this phenomenon. Specific regions of the C-termini were selected on the basis of putative sites of posttranslational modifications and predicted structural features. We generated models of the structure of the C-termini of the receptors on the basis of the structure of the sequence-related C-terminus of the BK₂ receptor [17]. On the basis of these structural features, we focused our efforts on the third (most C-terminal) α -helix of the C-termini (E3A and E3B). This helix is highly charged and does not display the amphipathic nature of the other two helices. We hypothesize that the first and second helix would associate with the membrane and therefore less likely to form multiple or high-affinity interactions with the cytoplasmic face of the receptors.

To empirically demonstrate these interactions, the amino acid sequence of the E3A and E3B helices were stably expressed in CHO cells transfected with either ET_A or ET_B receptors. CHO cells do not natively express either receptor [31]. Here we examined the effect of these signaling motifs on both homoreceptor and heteroreceptor downstream signaling. The biological action of these peptides in combination with the activation of the receptors by ET-1 proved dramatic. There is a clear interaction between the E3B peptide and the ET_A receptor; an interaction resulting in a 22-fold enhancement of ERK phosphorylation. Additionally, the ET_B peptide combined with the ET_B receptor and the ET_A peptide

combined with the ET_A receptor decreased ET-1 activation of ERK. These results illustrate heterologous ET-1 receptor activation and homologous receptor interference.

For the molecular modeling efforts, carried out to examine possible interactions between the receptors and the C-terminus derived peptides, we concentrated on E3B as it produced the largest alteration in signaling. From the docking, E3B is found to associate with the cytoplasmic domain of the ET_A receptor, binding between the helical domains of the C-terminus and the small IC2. Interestingly, no such binding mode was found for E3B with the ET_B receptor. These findings suggest that E3B is competing with ET_B for coupling to proteins in the signaling pathway and therefore antagonizing the ET-1 signaling (Figure 3). In contrast, the association of E3B with the cytoplasmic region of the ET_A, leads to a significant enhancement of the ET-1-induced signaling. Of course, this hypothesis is based on the homology modeling of the receptors cytoplasmic domains and requires experimental verification using site-directed mutagenesis and examination of receptor chimera; experiments which are ongoing. Based on simple sequence comparison, there are a number of differences in the cytoplasmic faces of ET_A and ET_B. Although the IC2 are very similar in ET_A and ET_B, a glutamine (Gln194) is replaced with a lysine (ET_A → ET_B), and in the E3B/ET_A complex (Figure 5) Gln194 is forming hydrogen bonds with Ser440 and Ser442 of E3B. Similarly, the second helix of the C-terminus of ET_B is more positively charged than that of ET_A, and therefore is less conducive to E3B binding. In the complex (Figure 5), E3B has Arg334 and Lys338 projecting towards the second helix in the C-terminus.

Future experiments combining cell permeable copies of receptor signaling motifs and cDNA stable expression of these sequences could become a basic as well as clinical tool to pinpoint receptor signaling action.

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Figure 1. Superposition of the structure of the C-terminus of the BK₂ receptor, as determined by NMR (gray), with the homology models of the C-termini of the endothelin A (dark blue) and endothelin B (light blue) receptors. The residues of the third α -helix of the C-termini of the endothelin A and endothelin B receptors are denoted.

Figure 2. Molecular models of the endothelin A (left) and endothelin B (right) receptors incorporating the structural features of the C-termini after energy minimization and molecular dynamics. The C-termini are colored as gray ribbons with side chains displayed.

Figure 3.

The relative cellular presence of pERK after stimulation with endothelin-1 of stably transfected CHO cells expressing the (A) endothelin A (CHO-ET_A); or (B) endothelin B (CHO-ET_B) receptors and either E3A or E3B C-terminal peptides. The level of pERK is shown relative to the control CHO-ET_A or CHO-ET_B cells, transfected with an empty vector (Emp Vec) not expressing the peptides. All tested cell cultures were treated with endothelin-1 at 10 nM (a maximal stimulatory dose) for 5 min (optimum incubation time). Blots are representative of three independent experiments. Bar graphs represent the mean value of relative ERK activation compared with the control (empty vector) cells. Error bars represent the standard deviation. Data were evaluated for statistical significance by use of a student's *t*-test. * = *P* < 0.05 versus Emp Vec.

Figure 4.

The low energy conformations from molecular docking of E3B to the endothelin A (left) and endothelin B (right) receptors. The C-terminus, second intracellular loop (IC2) and third intracellular loop (IC3) of the receptors are color-coded in gray, pink, and light green, respectively.

Figure 5.

Expanded view (from cytoplasmic face) of the interaction between the cytoplasmic domain of the endothelin A receptor and the E3B peptide. The endothelin receptor is blue, with the C-terminus, the second intracellular loop(IC2), and the third intracellular loop (IC3) color-coded in gray, pink, and light green, respectively. The E3B peptide is colored in yellow, with the side chains colored by heteroatom. Some of the key residues (Q194, R334, and K338) discussed in the text are highlighted.