A consensus sequence in the endothelin-B receptor second intracellular loop is required for NHE3 activation by endothelin-1

Kamel Laghmani,1 Aiji Sakamoto,2 Masashi Yanagisawa,3 Patricia A. Preisig,1,* and Robert J. Alpern1,*

1Department of Internal Medicine and the 3Howard Hughes Medical Institute, University of Texas Southwestern Medical Center, Dallas, Texas; and 2Division of Biotechnology and Department of Bioscience, National Cardiovascular Center Research Institute, Osaka, Japan

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Laghmani, Kamel, Aiji Sakamoto, Masashi Yanagisawa, Patricia A. Preisig, and Robert J. Alpern. A consensus sequence in the endothelin-B receptor second intracellular loop is required for NHE3 activation by endothelin-1. Am J Physiol Renal Physiol 288: F732–F739, 2005. First published December 14, 2004; doi:10.1152/ajprenal.00300.2004.—Endothelin-1 (ET-1) increases the activity of Na+/H+ exchanger 3 (NHE3), the major proximal tubule apical membrane Na+/H+ antiporter. This effect is seen in opossum kidney (OKP) cells expressing the endothelin-B (ETB) and not in cells expressing the endothelin-A (ETα) receptor. However, ET-1 causes similar patterns increases in cell [Ca2+]i in ETα- and ETβ-expressing OKP cells, implying that an additional mechanism is required for NHE3 stimulation by the ETB receptor. The present studies used ETα and ETB receptor chimeras and site-directed mutagenesis to identify the ET receptor domains that mediate ET-1 regulation of NHE3 activity. We found that binding of ET-1 to the ETA receptor inhibits NHE3 activity, an effect for which the COOH-terminal tail is necessary and sufficient. ET-1 stimulation of NHE3 activity requires the COOH-terminal tail and the second intracellular loop of the ETB receptor. Within the second intracellular loop, a consensus sequence was identified, KXXXVPKXXXV, that is required for ET-1 stimulation of NHE3 activity. This sequence suggests binding of a homodimeric protein that mediates NHE3 stimulation.

opossum kidney cells; endothelin-A/endothelin-B chimeras; sodium/hydrogen antiporter activity

THE ENDOTHELINS (ET) are a family of three 21-amino acid peptides, ET-1, ET-2, and ET-3. They are involved in a variety of physiological responses, such as vascular smooth muscle cell contraction and dilation, development, and regulation of renal function (17, 35). The biological effects of the ETs are mediated by two receptor subtypes, ETα and ETβ (3, 13, 32, 34). ET-1 and ET-2 bind the ETα receptor with high affinities, whereas ET-3 binds with low affinity. The three isopeptides bind the ETβ receptor with similar high affinities. Both receptors have seven hydrophobic transmembrane domains, characteristic of G protein-coupled receptors. Human ETα and ETβ receptors exhibit significant amino acid sequence identity (55% overall, 74% within the putative transmembrane helices) (33, 36). Of the receptor subdomains, the extracellular NH2 terminus and the intracellular COOH-terminal tail display the least sequence similarities, whereas the sequences in intracellular loops 2 and 3 are relatively similar. Despite their similarities, studies have demonstrated distinct roles for these intracellular loops in the human receptors. Human ETα and ETβ selectively couple to Gαq and Gαi, respectively, with the second and third intracellular loops of human ETα involved in Gαq activation and the third intracellular loop of human ETβ involved in Gαi activation (22).

ET-1 increases the activity of the proximal tubule apical membrane Na+/H+ antiporter, which mediates the majority of proximal tubular NaCl and NaHCO3 absorption (10, 12, 29, 30). This proximal tubule apical membrane Na+/H+ activity is mediated by NHE3 (1, 4). OKP cells, an opossum kidney cell line with many proximal tubule characteristics, express NHE3 (2, 23, 24). Using stable transfection, we established OKP cell lines expressing ETA and ETB receptors and showed that in cells expressing the ETB, but not the ETα receptor, ET-1 increases NHE3 activity (7). In addition, NHE3 activation by ET-1 was inhibited by ETB receptor blockers, but not by ETα receptor blockers. Finally, in proximal tubules harvested from ETB receptor knockout mice, ET-1 fails to increase NHE3 activity, whereas stimulation is seen in tubules from wild-type mice (20). These findings agree with immunohistochemistry and binding studies showing that the ETB receptor is the predominant ET receptor on the renal proximal tubule (9, 42). However, the mechanism responsible for the receptor specificity of NHE3 regulation remains unclear. We previously showed that ET-1 causes similar patterns of protein tyrosine phosphorylation, adenylyl cyclase inhibition, and increases in cell [Ca2+]i in ETα- and ETβ-expressing OKP cells, implying that an additional signaling pathway must mediate the receptor specificity of NHE3 regulation (7, 8, 28).

To address this, in the current study human ETα/ETB Receptor chimeras and site-directed mutagenesis were used to identify the ET receptor domain(s) involved in ET-1 regulation of NHE3 activity in OKP cells. We found that ETα receptor activation inhibits NHE3 activity, an effect for which the COOH-terminal tail is necessary and sufficient. Activation of NHE3 by ET-1 requires the COOH-terminal domain and the second intracellular loop of the ETB receptor. Site-directed mutagenesis within the ETB second intracellular loop identified residues critical for stimulation of NHE3 activity by ET-1. These residues define a consensus sequence, KXXXVPKXXXV, required for ET-1 stimulation of NHE3.

METHODS

Materials. All chemicals were obtained from Sigma (St. Louis, MO) unless otherwise noted as follows. Penicillin and streptomycin

* P. A. Preisig and R. J. Alpern contributed equally to these studies.

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were from Whittaker MA Bioproducts (Walkersville, MD); BCECF-AM was from Molecular Probes (Eugene, OR); ET-1 was from Peptides International (Louisville, KY); and 125I-ET-1 was from Amersham (Arlington Heights, IL).

**Cell culture and transfections.** OKP cells were grown in DMEM supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a CO2 incubator. To overexpress human ETA and ETB receptors, cells were transiently transfected with wild-type, chimera, or mutant receptors using the PME18S expression vector driven by a constitutively active SRα promoter (18). OKP cells were plated on coverslips in 35-mm plastic dishes, grown to 80% confluence, transfected with 1.1 μg DNA using Lipofectamine Plus according to the manufacturer’s instructions, and grown to 100% confluence. Confluent cells were rendered quiescent by the removal of serum for 24 h before study. Cells on control and experimental coverslips were derived from the same flask and passage and were studied on the same day.

**Stratagene QuikChange Site-Directed Mutagenesis Kit (La Jolla, CA),** was designed to contain the desired mutation (Table 1). The plasmid was PCR amplified from methylated, nonmutated parental DNA template. The circular mutated plasmid DNA was generated as described (33).

**Site-directed mutagenesis.** Point mutations were generated using a Stratagene QuikChange Site-Directed Mutagenesis Kit (La Jolla, CA), as per kit protocol. Two primers complementary to each other were designed, which were acidified with nigericin and the initial rate of Na-dependent intracellular alkalinization was measured, as previously described (7, 8, 28). In all studies, control and experimental cells were from the same passage and were assayed on the same day. Exposure to 10⁻⁸ M ET-1 was begun at the time of BCECF loading (30 min before study). Control cells were exposed to the vehicle (0.1% acetic acid).

**RESULTS**

We previously showed in stably transfected cells that the ET-1-induced increase in NHE3 activity is mediated by ETB and not ETA receptors. The first step of this study was to compare the effect of ET-1 on NHE3 activity in wild-type OKP cells and OKP cells transiently transfected with the ETB or ETA receptor cDNA. In wild-type untransfected cells, 10⁻⁸ M ET-1 applied for 30 min caused a consistent small, but statistically insignificant, increase (+15%) in NHE3 activity (Fig. 1). When the ETA receptor was expressed, 10⁻⁸ M ET-1 significantly increased NHE3 activity by 38%, in agreement with our previous study (7). In contrast, in OKP cells expressing the ETA receptor, 10⁻⁸ M ET-1 significantly inhibited NHE3 activity (−15%).

**Statistics.** Data are reported as means ± SE. Statistical significance was determined using a paired Student’s t-test (NHE3 activity studies) or ANOVA (binding studies) and set at P < 0.05.

**RESULTS**

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**Table 1. Sequences of primers**

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<th>Mutation Name</th>
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**RESULTS**

We previously showed in stably transfected cells that the ET-1-induced increase in NHE3 activity is mediated by ETB and not ETA receptors. The first step of this study was to compare the effect of ET-1 on NHE3 activity in wild-type OKP cells and OKP cells transiently transfected with the ETB or ETA receptor cDNA. In wild-type untransfected cells, 10⁻⁸ M ET-1 applied for 30 min caused a consistent small, but statistically insignificant, increase (+15%) in NHE3 activity (Fig. 1). When the ETA receptor was expressed, 10⁻⁸ M ET-1 significantly increased NHE3 activity by 38%, in agreement with our previous study (7). In contrast, in OKP cells expressing the ETA receptor, 10⁻⁸ M ET-1 significantly inhibited NHE3 activity (−15%).
To identify the domain(s) of the ET<sub>B</sub> and ET<sub>A</sub> receptors involved in ET-1 regulation of NHE3 activity, we transfected cells with chimeric ET<sub>A</sub> and ET<sub>B</sub> receptor cDNAs. Figure 2 shows the restriction sites used to generate the chimeric cDNAs. Nomenclature will indicate the specific domains derived from each receptor. For example, chimera A(N-III)B(IV-C) designates a chimeric receptor consisting of the ETA sequence from the NH<sub>2</sub>-terminal extracellular tail to the third transmembrane domain followed by the ETB sequence from the second intracellular loop through the COOH-terminal tail. To generate this chimera wild-type ETA and ETB receptor, cDNA was cut with BssHII (Fig. 2) (33). OKP cells were transiently transfected with each chimeric cDNA and NHE3 activity was measured following exposure to 10<sup>-8</sup> M ET-1 or vehicle.

**Role of the COOH-terminal domain.** Experiments shown in Fig. 3 examined the role of the COOH-terminal domain. Again, activation of the ETA receptor led to inhibition of NHE3 activity (Fig. 3). Substitution of the ETA COOH-terminal tail with the ETB COOH-terminal tail [A(N-VII)B(C)] prevented this effect, whereas substitution of the ETB COOH-terminal tail with the ETA COOH-terminal tail [B(N-VII)A(C)] mediated inhibition (26%, *P* < 0.01). Thus the presence of the COOH-terminal domain of the ETA receptor is necessary and sufficient to mediate inhibition of NHE3 activity by ET-1.

By contrast, the above results using the chimera A(N-VII)B(C) suggest that the ETB COOH-terminal domain is not sufficient to mediate stimulation of NHE3 activity by ET-1. The failure of B(N-VII)A(C) to mediate ET-1-induced stimulation of NHE3 could indicate a requirement for the COOH terminus or could be due to ETA-induced inhibition mediated by the ETA COOH terminus. To determine whether the ETB COOH-terminal tail is required for ET-1 stimulation of NHE3, we examined a truncated ETB receptor in which the COOH-terminal tail was removed (18). As shown in Fig. 3, with removal of the COOH-terminal tail ET-1 was without effect on NHE3 activity. Taken together, these results suggest that the ETB COOH-terminal tail is necessary but not sufficient to mediate stimulation of NHE3 activity by ET-1.

**Role of the ETB receptor intracellular loops.** To identify other key domains of the ETB receptor required for ET-1 stimulation of NHE3 activity, we used a series of chimeras in which domains of the ETB receptor were replaced with the corresponding region of the ETA receptor. As shown in Fig. 4, when the ETB sequence extended from the COOH terminus to the third intracellular loop, [A(N-V)B(VI-C) and A(N-IV)B(V-C)], ET-1 had no effect on NHE3 activity. By contrast, when the second intracellular loop of the ETB receptor was included [A(N-III)B(IV-C)], ET-1 stimulation of NHE3 activity was restored (+35%). These results suggest that in addition to the COOH-terminal tail, a specific region including transmem-
brane helix III and/or the second intracellular loop of the ETB receptor is required for ET-1 stimulation of NHE3 activity.

To confirm this, an additional series of chimeras were used (Fig. 5). When the sequence extending from transmembrane helices III to VI of the ETB receptor was replaced with the corresponding ET A sequence [B(N-III)A(IV-VI)B(VII-C)], ET-1 was without effect on NHE3 activity. When only the region between transmembrane helices V and VI of the ETB receptor was replaced with the corresponding ETA sequence [B(N-IV)A(V-VI)B(VII-C)], ET-1 stimulation of NHE3 activity was restored (+36%). Together, these data confirm that the domain containing the second intracellular loop and transmembrane helix IV of the ETB receptor is critical in mediating ET-1-induced NHE3 activation.

To further confirm this conclusion, we used a chimera in which both the second intracellular loop and COOH-terminal tail were derived from the ETB receptor and all other intracellular sequences were from the ETA receptor [A(N-III)B(IV-V)A(VI-VII)B(C)]. As shown in Fig. 5, in cells expressing this chimera ET-1 increased NHE3 activity by 35%, confirming that the second intracellular loop and COOH-terminal tail of ETB are both required and sufficient for ET-1 stimulation of NHE3 activity.

Amino acid residues in the ETB receptor second intracellular loop required for ET-1 stimulation of NHE3 activity. The above studies all confirmed a key role for the domain including the second intracellular loop and transmembrane helix IV in NHE3 stimulation. We reasoned that the key domain was most likely intracellular and further pursued the second intracellular loop. The second intracellular loop is highly conserved with only six amino acids that differ between the ETA and ETB receptors (Fig. 6). The specific role of each of these amino acids was analyzed by site-directed mutagenesis converting each of the six ETB amino acids individually to their corresponding ETA receptor amino acid (Fig. 6 and Table 1). As shown in Fig. 7, in cells expressing wild-type ETB receptor, ET-1 increased NHE3 activity by 46% (P < 0.001). Individual mutation of ETB residues I836 and W844 to the corresponding ETA residues V835 and V843, respectively, did not prevent this effect (ET-1 increased NHE3 activity 44 and 52%, respectively, both P < 0.001). By contrast, when ETB receptor residues K837, V841, K843, and V847 were individually replaced with the corresponding ETA receptor residues (Q836, I840, L842, and I846, respectively), the ETB receptor lost its ability to mediate ET-1 stimulation of NHE3 activity (Fig. 7). Thus ETB...
Fig. 7. Role of amino acid residues in the second intracellular loop that differ between the ET<sub>A</sub> and ET<sub>B</sub> receptors. Cells were grown, treated, and assayed as in Fig. 1. Mutations were made as indicated in Table 1. ET<sub>A</sub>: n = 21; I836V: n = 7; K837Q: n = 9; V841I: n = 9; K843L: n = 10; W844V: n = 11; V847I: n = 8. *P < 0.01.

receptor residues K<sup>837</sup>, V<sup>841</sup>, K<sup>843</sup>, and V<sup>847</sup> play a critical role in ET-1 stimulation of NHE3 activity, whereas I<sup>836</sup> and W<sup>844</sup> are not required.

The two pairs of K and V residues (K<sup>837</sup> and V<sup>841</sup>, K<sup>843</sup> and V<sup>847</sup>) that were found to be required for ET-1 stimulation of NHE3 activity are each separated by three amino acids. We next tested the role of the six amino acid residues (G<sup>838</sup>, I<sup>839</sup>, NHE3 activity are each separated by three amino acids. We statistically different between cells expressing the wild-type activity (Table 2). As shown, specific ET-1 binding was not mutared receptors in which ET-1 did not stimulate NHE3 activity. Specific ET-1 binding was not a consequence of a difference in receptor expression or failure of ET-1 to bind to a mutated receptor, ligand binding studies were performed in wild-type OKP cells and OKP cells expressing intact, and key truncated, chimeric, or mutated receptors in which ET-1 did not stimulate NHE3 activity (Table 2). As shown, specific ET-1 binding was not statistically different between cells expressing the wild-type ET<sub>B</sub> receptor and those expressing key truncated, chimeric, or mutated receptors.

DISCUSSION

The ETs are a family of three conserved 21 amino acid peptides that bind to and mediate their effects through two G protein-coupled receptor subtypes, ET<sub>A</sub> and ET<sub>B</sub> (3, 13, 32). Despite significant amino acid homology, the receptors can be pharmacologically distinguished by their affinity for the three ET isopeptides and their distinct physiological roles (22, 26, 36). The ET<sub>B</sub> receptor mediates release of relaxing factors causing vasodilation and is involved in inflammatory pain, whereas the ET<sub>A</sub> receptor mediates vasoconstriction, stimulates mitogenesis and matrix formation, is antiapoptotic, and is involved in acute or neuropathic pain (26). Typically, the ET<sub>B</sub> receptor activates G<sub>q</sub>, whereas the ET<sub>A</sub> receptor activates G<sub>q</sub>5, but this G protein coupling is not fixed in all cells (22, 36).

We previously showed that ET-1 stimulates NHE3 activity in cultured OKP cells, an effect that is mediated through the ET<sub>B</sub> and not the ET<sub>A</sub> receptor (7, 19). ET-1/ET<sub>B</sub> stimulation of NHE3 activity involves NHE3 phosphorylation and exocytic insertion of NHE3 into the apical membrane (27, 28). As shown in Fig. 1, ET-1/ET<sub>A</sub> signaling not only fails to stimulate NHE3 activity but inhibits the transporter. The physiological significance of NHE3 inhibition is unclear as ET-1 stimulates the proximal tubule Na<sup>+</sup>/H<sup>+</sup> antiporter, an effect mediated by the ET<sub>B</sub> receptor (19, 20).

Because of these differing effects on NHE3, we initially sought to determine signaling pathways that were differentially activated by the ET<sub>B</sub> and ET<sub>A</sub> receptors in OKP cells. Using inhibitors, we found that NHE3 activation by the ET<sub>B</sub> receptor was mediated by increases in cell Ca<sup>2+</sup> and activation of Ca<sup>2+</sup>-calmodulin kinase and tyrosine kinases. On ET-1 addition, both receptors were found to increase cell Ca<sup>2+</sup> with patterns that were indistinguishable. In addition, activation of ET-1 binding to receptors. To address the possibility that the varying effect of ET-1 on NHE3 activity in the above studies was a consequence of a difference in receptor expression and/or failure of ET-1 to bind to a mutated receptor, ligand binding studies were performed in wild-type OKP cells and OKP cells expressing intact, and key truncated, chimeric, or mutated receptors in which ET-1 did not stimulate NHE3 activity (Table 2). As shown, specific ET-1 binding was not statistically different between cells expressing the wild-type
both receptors resulted in tyrosine phosphorylation of proteins with a pattern consistent with focal adhesion protein phosphorylation. Finally, although activation of ETA and ETB receptors can have opposing effects on adenyl cyclase in many cells, in OKP cells both receptors inhibited adenyl cyclase (7, 28). These findings led us to hypothesize that an additional signaling pathway must be required for ETB receptor activation of NHE3, perhaps a protein that binds selectively to the ETB receptor. To address this, the present studies were designed to define domains unique to the ETB receptor that were required for NHE3 activation.

The ETA and ETB receptors exhibit 55% overall amino acid homology (22, 36). Within the receptor domains, the least homology is seen in the intracellular COOH-terminal domain and the most homology is associated with the transmembrane helices. In addition, intracellular loops, a likely site of protein binding, are highly homologous. In the present studies, OKP cells were transiently transfected with human ETA and ETB receptor chimeras as well as mutated ETB receptor cDNAs to determine the domains and sequences responsible for ET-1/ETB stimulation of NHE3. Our laboratory previously showed that we achieve ~80% transfection efficiency with transient transfection in OKP cells (14). In addition, we completely inhibited a number of cellular processes in cells transiently transfected with dominant-negative constructs, again suggesting high transfection efficiency (21, 39, 40).

The studies led to a number of conclusions. First, the COOH-terminal tail of the ETA receptor is necessary and sufficient for inhibition of the NHE3 activity by ET-1. Thus a chimeric receptor that contains only the COOH-terminal tail of ETA, with the remainder of the receptor derived from the ETB receptor, inhibits NHE3. Similarly, an ETA receptor in which the COOH-terminal domain has been replaced with the relevant ETB sequence loses the ability to inhibit NHE3 activity.

Second, the COOH-terminal tail of ETB is required for stimulation of NHE3. Deletion of this sequence eliminates regulation, and replacement with the corresponding ETA sequence leads to ET-1-induced inhibition of NHE3 activity. However, unlike results with the ETA receptor, the COOH-terminal tail is not sufficient for stimulation of NHE3. The chimera containing the COOH-terminal ETB sequence with the remainder of the protein derived from the ETA receptor fails to regulate NHE3 activity. This suggests that in addition to the COOH-terminal tail, at least one other sequence is required for NHE3 activation by ET-1/ETB.

The role of the COOH-terminal tail of G protein-coupled receptors has not been studied extensively. In the parathyroid Ca2+-sensing receptor, individual point mutations of His830 and Phe882 in the COOH-terminal tail to alanine residues were associated with decreased total inositol phosphate production and retention of the receptors in the endoplasmic reticulum, suggesting that the COOH-terminal tail is required for efficient targeting of the receptor to the cell surface (6). In the present studies, however, absence of the ETB COOH-terminal tail did not decrease ET-1 binding, demonstrating that trafficking to the plasma membrane had not been impaired.

In addition to a requirement for the COOH-terminal tail, NHE3 activation by ET-1/ETB required a sequence within the second intracellular loop. Comparing a series of chimeras, results consistently demonstrated that the presence of sequences in the second intracellular loop and transmembrane domain IV, between the BstHI and NcoI restriction sites, was required for NHE3 activation. Mutational studies localized the key sequences to the second intracellular loop and further defined the specific sequence required: two essential lysine (K) and two essential valine (V) residues that are spaced as follows: KXXXVPKXXXV (Fig. 6). Both the spacing between the lysine and valine residues and the proline (P) residue were found to be essential to confer ET-1 stimulation of NHE3. The presence of the repeat sequence (KXXXV) and the space requirement for three residues between the K and V pairs.

### Table 2. ET-1 binding to receptors

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<td>B(N-III)A(IV-VII)B(VII-C)</td>
<td><img src="image" alt="B(N-III)A(IV-VII)B(VII-C)" /></td>
<td>114.2 ± 6.7</td>
</tr>
<tr>
<td>KB37Q</td>
<td><img src="image" alt="KB37Q" /></td>
<td>96.3 ± 5.6</td>
</tr>
<tr>
<td>V841I</td>
<td><img src="image" alt="V841I" /></td>
<td>125.7 ± 13.8</td>
</tr>
<tr>
<td>K843L</td>
<td><img src="image" alt="K843L" /></td>
<td>118.9 ± 5.0</td>
</tr>
<tr>
<td>V847I</td>
<td><img src="image" alt="V847I" /></td>
<td>131.4 ± 13.2</td>
</tr>
<tr>
<td>Delete G838</td>
<td><img src="image" alt="Delete G838" /></td>
<td>95.5 ± 7.2</td>
</tr>
</tbody>
</table>

Values are means ± SE. Cells were grown and treated as in Fig. 1. Ligand binding was assayed as described in METHODS. Wild-type opossum kidney cells: n = 9; endothelin (ETb) cells: n = 6; ETA cells: n = 6; ETB minus C-tail; n = 6; A(N-V)B(VI-C); n = 3; B(N-III)A(IV-VII)B(VII-C); n = 3; KB37Q; n = 3; V841I: n = 6; K843L: n = 6; V847I: n = 6; Delete G838: n = 3.
suggest that this portion of the receptor may be involved in the binding of a homodimer to the receptor. The presence of the proline residue, typically associated with flexibility in a protein, between the two repeat sequences would also facilitate binding of a homodimer.

The second and/or third intracellular loops have been shown to be critical for signaling in other G protein-coupled receptors. The second intracellular loop of the thrombin receptor confers specificity to Gq coupling, is hypothesized to be the relevant domain involved in G protein coupling and to mediate conformational changes that prevent or promote G protein coupling in the muscarinic receptor, and is critical for activating the phospholipase C (PLC) pathway through the Gq α-subunit in the PTH/PTH-related protein signaling pathway (5, 15, 25, 41). The second and third intracellular loops are involved in inhibiting angiotensin-dependent activation of PLC via the angiotensin Ia receptor (38). The second intracellular loop of the metabotropic glutamate receptor 1 is critical for signal transduction, but unlike most other G protein-coupled receptors, requires cooperation with another intracellular domain (11). In Chinese hamster ovary cells expressing ET\(_A\)/ET\(_B\) receptor chimeric proteins, intracellular loops 2 and/or 3 of the ET\(_A\) receptor mediate ET-1-induced stimulation of cAMP formation, whereas intracellular loop 3 of the ET\(_B\) receptor mediates ET-1-induced inhibition of cAMP formation, demonstrating that intracellular loops 2 and 3 direct signaling through Gαs and Gαi, respectively (36). Thus it appears that the intracellular loops of G protein-coupled receptors mediate signal transduction. In the present studies, we show that intracellular loop 2 of the ET\(_B\) receptor mediates ET-1 stimulation of NHE3 activity.

The lack of an effect on ET-1 binding to the chimeric and mutated receptors is consistent with studies in COS-7 cells (33). Similarly, the lack of an effect on ET-1 binding with removal of the ET\(_B\) COOH-terminal tail is consistent with studies from Sakamoto et al. (18) showing that the COOH-terminal tail of the ET\(_B\) receptor is involved with signaling but not ligand binding. These observations suggest that the tertiary structure of the ET\(_B\) receptor has been maintained in the chimeric and mutated receptors and that failure of ET-1 to stimulate NHE3 activity is not due to failure of the chimeric or mutated receptors to be expressed on the apical membrane.

In many of the studies in which cells were transfected with either the ET\(_A\) receptor or one of the inactive receptors, baseline NHE3 activity was observed to be increased, compared with cells transfected with the ET\(_B\) receptor or a functional receptor. One possible explanation for this observation is that the vacant ET\(_A\) receptor stimulates NHE3 activity and/or the vacant ET\(_B\) receptor inhibits NHE3 activity, both effects due to binding of a NHE3-regulatory protein. Binding of ET-1 to these receptors could then inhibit binding of the regulatory protein, relieving NHE3 regulation. Thus binding of ET-1 to the ET\(_A\) receptor may prevent NHE3 inhibition and/or binding of ET-1 to the ET\(_B\) receptor may prevent NHE3 stimulation by the vacant receptor.

Receptors have two functions, ligand recognition and binding ("address" function) and signal transmission ("message" function). Ligand binding studies have proposed that the region of transmembrane helices IV-VI and the adjacent loops of the ET\(_A\) and ET\(_B\) receptors determine the selection of ligand-receptor interaction and thus are considered to be the address recognition subdomain (33). In the case of the ET\(_A\) receptor, this region matches more closely to the NH\(_2\) terminus (address domain) of ET-1 compared with ET-3, and thus ET\(_A\) binds ET-1 with higher affinity than ET-3 (33). Subtle differences in the ET\(_B\) receptor allow the address recognition domain to interact with a wider spectrum of ligand address domains, making the ET\(_B\) receptor more promiscuous (33). The COOH-terminal portion of the ligand interacts with transmembrane helices I, II, III, and VII of the ET\(_A\) and ET\(_B\) receptors to transmit the ligand message (22). The present studies demonstrate that the amino acid sequence in the second intracellular loop and first part of transmembrane helix IV transmit the signal mediating ET-1 stimulation of NHE3 activity. The combination of a required repeat sequence, required spacing between essential amino acid residues, and a required proline residue between the repeat sequences suggests that the protein that binds the receptor might have symmetrical "arms" that interact with the receptor. Such a protein could be either a single protein or a homodimer.

In summary, the present studies identify the COOH-terminal tail and a sequence within the second intracellular loop of the ET\(_B\) receptor that are necessary and sufficient to mediate ET-1 stimulation of NHE3 activity. The presence of a repeat sequence with specific space requirements and a required proline residue separating the repeat sequences suggests that signal transmission is mediated by the binding of a homodimeric protein to the receptor.

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