Mutational Analysis of the Conserved Asp<sup>2.50</sup> and ERY Motif Reveals Signaling Bias of the Urotensin II Receptor

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Received January 8, 2008; accepted May 22, 2008

ABSTRACT

Class A (rhodopsin-like) G protein-coupled receptors possess conserved residues and motifs that are important for their specific activity. In the present study, we examined the role of residue Asp<sup>97.50</sup> as well as residues Glu<sup>147.49</sup>, Arg<sup>148.50</sup>, and Tyr<sup>149.51</sup> of the ERY motif on the functionality of the urotensin II receptor (UT). Mutations D<sup>97.50</sup>A, R<sup>148.50</sup>A, and R<sup>148.50</sup>H abolished the ability of UT to activate phospholipase C, whereas mutations E<sup>147.49</sup>A and Y<sup>149.51</sup>A reduced the ability to activate PLC by 50%. None of the mutants exhibited constitutive activity. However, R<sup>148.50</sup>A and R<sup>148.50</sup>H promoted ERK1/2 activation, which was abolished by 4-(3-chloroanilino)-6,7-dimethoxyquinazoline (AG1478), an inhibitor of epidermal growth factor receptor (EGFR) tyrosine kinase activity. Both these mutants were capable of directly activating EGFR, which confirmed that they activated the mitogen-activated protein kinase (MAPK) pathway by a G<sub>q/11</sub>-independent transactivation of EGFR. The D<sup>97.50</sup>A, R<sup>148.50</sup>A, and R<sup>148.50</sup>H mutants did not readily internalize and did not promote translocation or colocalize with β-arrestin2-GFP. Finally, the agonist-induced internalization of the E<sup>147.49</sup>A mutant receptor was significantly increased compared with wild-type receptor. This study highlights the major contribution of the conserved Asp<sup>2.50</sup> residue to the functionality of the UT receptor. The Arg residue in the ERY motif of UT is an important structural element in signaling crossroads that determine whether G<sub>q/11</sub>-dependent and -independent events can occur.

GPCRs, which make up the largest family of membrane proteins, are coded by more than 720 genes in the human genome (Wise et al., 2004). Of these, 280 belong to family A, which are classified by homology with rhodopsin (http://www.iupharbb.org/receptorList/results.php). Class A GPCRs respond to a wide array of ligands ranging from photons to large glycoproteins (Bockaert and Pin, 1999). More than 25% of clinically marketed drugs currently target class A GPCRs (Overington et al., 2006). In view of the broad range of biological functions and the great potential for pharmacological intervention, considerable interest has been generated in understanding the mechanisms by which GPCRs mediate their effects.

Overall homology among class A receptors is low and is restricted to a number of highly conserved key residues, suggesting that these residues play an essential role in the structural and/or functional integrity of these receptors. Most members of the rhodopsin-like family possess a conserved Asp residue (D<sup>2.50</sup>) in transmembrane domain 2 (TMD2) and a Glu/Asp-Arg-Tyr (E/D<sup>3.49</sup>-R<sup>3.50</sup>-Y<sup>3.51</sup>) motif at the junction of TMD3 and the second intracellular loop. Using different biochemical methods, several groups have demonstrated movements between TMD3 and TMD6 when receptors undergo activation (Gether et al., 1997). In rhodopsin, the basal inactive state is maintained by an ionic lock, where Arg<sup>3.50</sup> and Glu<sup>3.49</sup> form a network of ionic interactions with Glu<sup>6.30</sup> at the cytoplasmic end of TMD6 (Palczewski et al., 2000). Protonation of Glu<sup>3.49</sup> restricts to a number of highly conserved key residues, suggesting that these residues play an essential role in the structural and/or functional integrity of these receptors. Most members of the rhodopsin-like family possess a conserved Asp residue (D<sup>2.50</sup>) in transmembrane domain 2 (TMD2) and a Glu/Asp-Arg-Tyr (E/D<sup>3.49</sup>-R<sup>3.50</sup>-Y<sup>3.51</sup>) motif at the junction of TMD3 and the second intracellular loop. Using different biochemical methods, several groups have demonstrated movements between TMD3 and TMD6 when receptors undergo activation (Gether et al., 1997). In rhodopsin, the basal inactive state is maintained by an ionic lock, where Arg<sup>3.50</sup> and Glu<sup>3.49</sup> form a network of ionic interactions with Glu<sup>6.30</sup> at the cytoplasmic end of TMD6 (Palczewski et al., 2000). Protonation of Glu<sup>3.49</sup>
in rhodopsin causes the pivoting movement between TMD3 and TMD6 and leads to receptor activation (Arnis et al., 1994). Mutations in Glu3.49 and Glu6.30 have been shown to promote constitutive activation of a number of GPCRs, suggesting that rhodopsin-like GPCRs may have similar activation mechanisms (Scheer et al., 1996; Ballesteros et al., 2001). However, replacement of Glu3.49 does not always lead to constitutive activity (Chung et al., 2002; Feng and Song, 2003; Capra et al., 2004). In addition, the crystal structure of the β2-adrenergic receptor indicates that in the basal state, the distance between TMD3 and TMD6 is greater in this receptor than in rhodopsin, which would not allow interactions between specific residues in these domains to occur (Rosenbaum et al., 2007). Discrepancies in the functional effects revealed by mutational analyses of these residues in class A GPCRs and those deduced from the crystal structure of the β2-adrenergic receptor suggest alternative roles for the E/D3.49 residue and the E/D/R/Y motif in class A GPCRs. For example, based on simulations using the α1B-adrenergic receptor, Scheer et al. (1996) suggested that Arg3.50 in the inactive state is constrained in a “polar pocket” formed by residues Asn3.50, Asp6.30, Asn7.49, and Tyr7.53.

Urotensin II (UII), a cyclic undecapeptide originally isolated from the caudal neurosecretory system of teleost fish, has since been identified in many classes of vertebrates, including humans (Douglas et al., 2004). Although it is clear that UII influences the physiological regulation of mammalian cardiovascular function, early studies led to conflicting reports of vasoactive responses, depending on the species or tissues studied, with UII acting both as a potent vasodilator (Stirrat et al., 2001) and a vasoconstrictor (Douglas et al., 2000). UII is the endogenous ligand for the G protein-coupled UT receptor (originally called GRPR) (Ames et al., 1999). The UT receptor belongs to the class A family of GPCRs. Upon UII binding, it functionally couples to Gαq/11, thereby promoting phospholipase C activation (Sætra and Opgaard et al., 2000), Ca2+ mobilization (Ames et al., 1999), and mitogen-activated protein kinase (MAPK) activation (Ziltener et al., 2002). UT is found in the central nervous system and is widely expressed in human tissues, including the left atrium and ventricle of the heart, smooth muscle cells in the coronary artery and aorta and endothelial cells in vascular beds (Douglas et al., 2004). Compelling evidence has implicated the urotensin system in the pathophysiology of hypertension, heart failure and of cardiac fibrosis and hypertrophy (Douglas et al., 2004).

Here, we investigate how substitution of the conserved residue Asp972.50 in TMD2 and Glu1473.49, Arg1483.50, and Tyr1493.51 in the ERY motif affects binding, signaling, and internalization of the UT receptor. Our results demonstrate that Asp2.50 plays a critical role in the activation of the UT receptor. Mutational analyses of the highly conserved Arg3.50 reveal that this residue is important in Gαq/11-protein-dependent UT signaling and reveals a Gαq/11-protein-independent activation of the MAPK pathway.

Materials and Methods

Materials. Bovine serum albumin and bacitracin were from Sigma-Aldrich (Oakville, ON, Canada). The cDNA encoding the rat UT receptor (GRPR) subcloned in the mammalian expression vector pcDNA3 was a generous gift from Dr. Brian O’Dowd (University of Toronto, Toronto, ON, Canada). Dubelco’s modified Eagle’s medium (DMEM), fetal bovine serum, penicillin-streptomycin, and oligonucleotide primers were from Invitrogen (Carlsbad, CA); FuGENE-6, protease inhibitor cocktail, and mouse monoclonal anti-my c antibody were from Roche Molecular Biochemicals (Mannheim, Germany); and rabbit polyclonal antibodies directed against phosphorylated and active forms of p42/p44 MAPK were from New England Biolabs (Mississauga, ON, Canada). Goat anti-mouse alkaline phosphatase-conjugated antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). Bisindolylmaleimide I (BIM), AG1478, mouse anti-phosphotyrosine (PY20) antibodies, and the phosphatase inhibitor cocktail set II were from Calbiochem (San Diego, CA) and urotensin II was from Phoenix Pharmaceuticals (Belmont, CA). 125I-UII (specific radioactivity, 400 Ci/mmol) was prepared with IODO-GEN as described by Fraker and Speck (1978). In brief, 25 μl of 1 mM peptide solution was incubated with 20 μg of IODO-GEN (Pierce Chemical Co., Rockford, IL) at 37°C for 30 min at room temperature. The labeled peptides were purified by HPLC on a C-18 column (10 μm; Alltech Associates Inc., Deerfield, IL) with a 35% to 45% acetonitrile gradient. The specific radioactivity of the labeled hormones was determined by self-deactivation and saturation binding analyses.

PCR Mutagenesis. Mutant UT receptor cDNAs were constructed by oligonucleotide-directed mutagenesis (Expand High Fidelity PCR System; Roche) using rat mycUT cDNA inserted into pcDNA3.1 (Invitrogen) as a template. Two sets of forward and reverse oligonucleotides were used to introduce mutations at Asp972.50, Glu1473.49, Arg1483.50, and Tyr1493.51. PCR products were subcloned using KpnI and XbaI sites of pcDNA3.1 after digestion by the same restriction enzymes. Mutagenesis was confirmed by automated nucleotide sequencing.

Cell Culture and Transfections. COS-7 cells were grown in DMEM containing 10% (v/v) fetal bovine serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin at 37°C. Cells (1.2 × 105) were plated in 100-mm culture dishes. When they reached 70% confluence, they were transfected with 2 μg of plasmid DNA and 4 μl of FuGENE-6 according to the manufacturer’s protocol. Transfected cells were grown for 48 h before performing binding assays. For confocal microscopy, HEK-293 cells were plated on 12-mm2 coverslips in six-well plates at a density of 2.5 × 105 cells/well. When cells reached 70% confluence, they were transfected with 1 μg of receptor plasmid DNA/0.5 μg of β-arrestin2-GFP plasmid DNA and 2 μl of FuGENE-6 according to the manufacturer’s protocol. Transfected cells were grown for 24 h, and images were captured using a Fluoview FV1000 confocal microscope (Olympus, Tokyo, Japan).

Binding Experiments. COS-7 cells were grown for 48 h after transfection in 100-mm culture dishes, washed once with PBS, and subjected to one freeze-thaw cycle. Broken cells were gently scraped in washing buffer (20 mM Tris-HCl, pH 7.4, and 5 mM MgCl2), centrifuged at 2500g for 15 min at 4°C and resuspended in binding buffer (20 mM Tris-HCl, pH 7.4, 5 mM MgCl2, 0.1% BSA, and 0.01% bacitracin). For saturation binding assays, broken cells (10–15 μg of protein) were incubated for 1 h at room temperature in binding buffer containing increasing concentrations of 125I-UII (0.15–20 nM, 50 Ci/mmol) in a final volume of 0.5 ml. Bound radioactivity was separated from free ligand by filtration through GF/C filters pre-soaked for at least 1 h in binding buffer. Nonspecific binding was measured in the presence of 1 μM unlabeled urotensin II. Receptor-bound radioactivity was evaluated by gamma counting. Kd and Bmax values were evaluated using Prism version 5.00 for Windows (Graph-Pad Software, San Diego, CA). Peptide stability was evaluated by thin layer chromatography. No degradation was observed after incubating the peptide with COS-7 cell membranes for up to 2 h.

Measurement of Agonist-Induced Inositol Phosphates. Inositol phosphate (IP) accumulation was measured as described previously (Lanctot et al., 1999). In brief, COS-7 cells were seeded in six-well plates, transfected, and labeled for 16 h in serum-free, ino-
sitol-free DMEM containing 10 μCi/ml of [myo-H]inositol (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK). Cells were washed twice with PBS supplemented with 0.1% dextrose and incubated in stimulation buffer (DMEM, 25 mM HEPES, pH 7.4, 10 mM LiCl, and 0.1% BSA) for 30 min at 37°C. Inositol phosphate production was induced by adding 0.1 μM urotensin II for 10 min at 37°C in stimulation buffer. Incubations were terminated by the addition of ice-cold perchloric acid (final 5% v/v). Water-soluble IPs were extracted with an equal volume of a 1:1 mixture of 1,2-trichlorotrifluoroethane and tri-n-octylamine. The samples were vigorously mixed and centrifuged at 250g for 30 min. The upper phase containing the IPs was applied to an AG1-X8 resin column (Bio-Rad Laboratories, Hercules, CA). The IPs were sequentially eluted with ammonium formate/formic acid solutions of increasing ionic strength. Fractions containing IPs were collected and radioactivity was measured using a liquid scintillation counter. Parallel plates were used to measure receptor expression and IP accumulation levels were adjusted based on receptor expression levels.

**Preparation of Cell Extracts and Immunoblotting.** Transfected COS-7 cells in 6-cm dishes were starved for 12 to 18 h in serum-free medium before stimulation. Where specified, protein kinase C inhibitor BIM alone or in combination with EGFR tyrosine kinase inhibitor AG1478 was added 30 min before UII. After stimulation, the medium was removed and cells were rinsed with ice-cold PBS. Cells were then lysed with 150 μl of radioimmunoprecipitation assay buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholic acid, 4 mM EDTA, and 1% SDS) containing protease inhibitor cocktail, 150 mM NaF, 50 mM sodium orthovanadate, and 250 μM β-glycerophosphate. Samples were centrifuged to remove insoluble material and protein concentrations were measured by Bradford assay. Proteins (40 μg) were resolved on 10% Tris/glycine polyacrylamide gels (Invitrogen) and transferred to nitrocellulose membranes for immunoblotting. Phosphorylated ERK1/2 and total ERK1/2 were detected by immunoblotting with key anti-rabbit horseradish peroxidase antibodies (Bethyl Laboratories, Montgomery, TX) using Western Lightning Chemiluminescence reagents, Montgomery, TX) using Western Lightning Chemiluminescence Reagent Plus according to the manufacturer’s instructions (PerkinElmer Life Sciences, Waltham, MA). Films were scanned, and band intensities were quantified using ImageJ software (http://rsb.info.nih.gov/ij/).

**EGFR Phosphorylation.** Transfected COS-7 cells in 10-cm dishes were starved for 12 to 18 h in serum-free medium before stimulation. Cells were then stimulated with UII (0.1 μM) or EGF (10 nM) for the indicated times, the medium was removed and cells were rinsed with ice-cold PBS. Cells were then lysed with 800 μl of lysis buffer (1% Triton X-100, 50 mM Tris, pH 7.5, 150 mM NaCl, and 4 mM EDTA) containing a protease inhibitor cocktail, 150 mM NaF, 50 mM sodium orthovanadate, and 250 μM β-glycerophosphate. Samples were centrifuged to remove insoluble material and protein concentrations were measured by Bradford assay. Equal amount of protein were immunoprecipitated using anti-EGFR antibodies. Western blots were probed sequentially with antiphosphotyrosine antibody (PY20) and the anti-EGFR antibody. The antibodies were visualized with donkey anti-rabbit horseradish peroxidase antibodies (Bethyl Laboratories, Montgomery, TX) using Western Lightning Chemiluminescence Reagent Plus according to the manufacturer’s instructions (PerkinElmer Life Sciences, Waltham, MA). Films were scanned, and band intensities were quantified using ImageJ software (http://rsb.info.nih.gov/ij/).

**Enzyme-Linked Immunosorbent Assay.** Receptor internalization was quantified by ELISA as described elsewhere (Parent et al., 2001). In brief, COS-7 cells were seeded at 1.2 × 10^5 cells/100-mm dish and transfected with 2 μg of myc-UT or mutant cDNA in combination with 2 μg of pcDNA3.1 or β-arrestin2-GFP cDNA. Twenty-four hours after transfection, cells were transfected into six wells of a 24-well plate. The following day, the cells were washed once with PBS and incubated in the presence or the absence of 0.1 μM UII for 120 min at 37°C in prewarmed DMEM supplemented with 0.5% BSA and 20 mM HEPES, pH 7.4. The medium was then removed, the cells were fixed in 3.7% formaldehyde/TBS (20 mM Tris, pH 7.4, 150 mM NaCl) for 5 min at room temperature and washed three times with TBS. Nonspecific binding was blocked by incubating the cells in 1% TBS/BSA for 30 min. Anti-myc-specific monoclonal antibody (1:500 in TBS/BSA) was then added, and the incubation was continued for a further 60 min. Cells were then washed three times with TBS, blocked in TBS/BSA for 15 min and incubated with alkaline phosphatase-conjugated goat anti-mouse (1:1000 in TBS/BSA) for 60 min. Cells were then washed three times with TBS. p-Nitrophenyl-phosphate (250 μl) was added, and the plates were incubated at 37°C until a yellow color appeared (standardized at 45 min). A 100-μl aliquot was collected, neutralized by adding 100 μl of 0.4 N NaOH, and the absorbance was read at 405 nm using a TiterTec Multiskan MCC/340 spectrophotometer. Cells transfected with pcDNA3.1 alone were used as controls. Results are shown as the percentage of cell surface receptor loss, where unstimulated cells represent 100% of receptor cell surface expression. To determine the expression levels of cell surface receptors, absorbance of unstimulated cells transfected with each receptor was measured and expressed as -fold absorbance over cells transfected with empty vector.

**Confocal Microscopy.** HEK-293 cells expressing mC-tagged UT (myc-UT) or mutant receptors and β-arrestin2-GFP were stimulated at 37°C with 0.1 μM UII for 30 min. They were then fixed for 30 min at room temperature in a solution of 3% (w/v) paraformaldehyde in PBS, permeabilized with 0.1% Triton X-100 for 10 min, and blocked with 1% BSA/PBS for 30 min. Cells were then incubated with anti-myc antibodies for 1 h at room temperature and then with Alexa Fluor 594-conjugated goat anti-mouse F(ab')2 for 1 h. Coverslips were mounted on slides using Vectashield mounting medium (Vector Laboratories, Peterborough, UK).

Cells were examined with a scanning confocal microscope (FV1000; Olympus, Tokyo, Japan) coupled to an inverted microscope with a PlanApo 60×/1.42 oil immersion objective. Specimens were laser-excited at 488 nm (40-mW argon laser) for GFP and 543 nm (helium-neon laser) for Alexa Fluor 594. To avoid cross-talk between GFP and Alexa Fluor 594 fluorescence, readings were recorded sequentially at wavelengths ranging from 505 to 530 nm and 580 to 650 nm, respectively. Serial horizontal optical sections (1024 × 1024 pixels) with two-time-line averaging were acquired. Olympus Fluoview software version 1.6a was used for image acquisition and analysis. The images were further processed using Adobe Photoshop (Adobe Systems, San Jose, CA).

**Data Analysis.** Results were collected in triplicate and are presented as means ± S.D. Binding curves, binding capacity (Bmax) and Kd values were determined using GraphPad Prism version 5.00 (GraphPad Software, San Diego CA). Statistical significance of the difference was assessed using one-way ANOVA and post hoc Dunnett test. *p < 0.05; ** p < 0.01; *** p < 0.001.

**Results**

**Binding Properties of Wild-Type and Mutant UT Receptors.** To study the involvement of Asp97 2.50, Glu147 3.49, Arg145 3.50, and Tyr149 5.51 in the functionality of the UT receptor, we mutated the residues to alanine (D97 2.50A, E147 3.49A, R148 3.50A, and Y149 5.51A, respectively). Another mutant (R148 3.50H), which was based on a reported single nucleotide polymorphism of the UT receptor (rs13305975), was also constructed. To facilitate the comparison of residues in TMDs, amino acids were indexed according to their positions relative to the most conserved residue in the TMD (designated X.50, X being the TMD number) in
which they are located (Fig. 1) (Ballesteros and Weinstein, 1995).

To characterize the pharmacological properties of all mutants, binding analyses using increasing concentrations of \(^{125}\)I-

\[ \text{UTII} \] (saturation kinetics) in transiently transfected COS-7 cells were used to characterize the pharmacological properties of the mutants. Table 1 shows that \(K_d\) values ranged from 1.4 ± 0.2 nM (E1473.49A) to 7.9 ± 2.3 nM (D972.50A), whereas expression levels ranged from 1.6 to 5.7 pmol/mg protein. ELISA analysis confirmed that mutant receptors were expressed at similar levels to those of wild-type receptor (Table 1).

**D972.50A and Arg1483.50 Mutants Abolish Inositol Phosphate Accumulation.** To evaluate the functional properties of the wild-type and mutant receptors, we tested their capacity to activate \(G_p\) by measuring UII-induced inositol phosphates accumulation. As shown in Fig. 2, 0.1 \(\mu\)M UII caused a significant inositol phosphate accumulation in COS-7 cells expressing the wild-type UT receptor. However, the effect of UII was significantly reduced in cells expressing the E1473.49A and Y1493.51A mutant receptors. Moreover, cells expressing the D972.50A or Arg1483.50 mutant receptors did not accumulate inositol phosphates after UII stimulation. No constitutive activity was observed for any receptor tested as measured by inositol phosphate accumulation under basal conditions (data not shown).

**Arg1483.50 Mutants Can Activate the MAPK Pathway.** UT receptor-mediated signaling activates the MAPK pathway, particularly by promoting extracellular-signal-regulated kinase 1/2 (ERK1/2) phosphorylation (Ziltener et al., 2002; Proulx et al., 2005). We previously reported that deleting specific residues in the carboxyl terminus of the UT receptor does not affect its ability to activate the ERK1/2 pathway (Proulx et al., 2005). In the present study, we measured the effect of Asp972.50 and ERY motif mutations on ERK1/2 activation by immunoblotting with antibodies directed against the phosphorylated active forms of ERK1/2 (p-ERK1/2). Addition of 0.1 \(\mu\)M UII to COS-7 cells transiently transfected with wild-type UT, E1473.49A, or Y1493.51A mutant receptors led to a robust activation of ERK1/2 after 2 and 30 min (Fig. 3, A and B). The D972.50A mutant did not activate ERK1/2 after UII stimulation. It is noteworthy that (unlike their inability to activate \(G_p\)) the R1483.50A and R1493.51H mutants were able to activate the MAPK pathway, although the temporal pattern of MAPK activation was altered. Indeed, very little ERK1/2 activation was observed after 2 min of UII stimulation whereas significant activation was detected after 30 min. The E1473.49A and Y1493.51A mutants exhibited a very slight but statistically significant increase of the basal phosphorylation of ERK1/2 that may represent constitutive activity of these mutants toward MAPK activation (Fig. 3B).

![Fig. 1. Schematic representation of the rat wild-type UT receptor (386 amino acids) and mutant receptors (light gray) in which Asp972.50, Glu1473.49, Arg1483.50, and Tyr1493.51 were replaced by alanine or histidine (R1483.50H). The most conserved residues of each TMD are circled in bold. Residues of the UT receptor that are important for UII binding are in dark gray (Boucard et al., 2003; Holleran et al., 2007), and those that are important for internalization are in black with white lettering (Proulx et al., 2005). Putative Asn-glycosylation sites (Asn29, Asn33) and the conserved disulfide bridge are depicted.](https://example.com/image)
UT-Dependent Activation of ERK1/2 Involves Both PKC and Transactivation of the EGF Receptor. Because the G protein-mediated activity of UII is typically transduced by Goq/11 leading to PKC activation, we evaluated the extent of PKC dependence on ERK1/2 activation in COS-7 cells transfected with the UT receptor. Moreover, EGFR-dependent ERK1/2 activation by UT receptor has been reported in cardiomyocytes (Onan et al., 2004). To determine the contribution of PKC- and EGFR-dependent mechanisms to ERK1/2 activation, cells were pretreated either with 1 μM BIM, a PKC inhibitor, or with 0.5 μM AG1478, an EGFR kinase inhibitor, or simultaneously with both inhibitors before evaluating the temporal pattern of ERK1/2 activation by UII. In untreated COS-7 cells transfected with the UT receptor, 0.1 μM UII promoted a specific temporal ERK1/2 activation pattern (Fig. 4, A and B). Early rapid activation of ERK1/2 was observed at 2 to 5 min, peaking at 5 min followed by a slow decrease toward basal levels (30 min). BIM-mediated PKC inhibition reduced early ERK1/2 activation but did not affect late ERK1/2 activation (10–30 min). On the other hand, AG1478 reduced ERK activation at each time point, but early activation (2–5 min) was maintained. The addition of both BIM and AG1478 almost entirely abolished the ability of the UT receptor to promote ERK1/2 phosphorylation, suggesting that the PKC- and EGFR-dependent pathways play a major role in ERK1/2 activation by UT. These results suggest that PKC activation and EGFR transactivation are the main ERK-activation pathways in COS-7 cells after UT receptor activation. They also revealed the important contribution of PKC to UII-induced phosphorylation of ERK1/2 at the early time points.

Arg1483.50 Mutants Transactivate EGFR via a Goq/11-Independent Mechanism. To better understand how the Arg1483.50 mutants promoted ERK1/2 phosphorylation, we determined the temporal pattern of ERK1/2 activation by R1483.50A and R1483.50H. Figure 4, C and D, showed that the Arg1483.50 mutants promoted ERK1/2 activation but that the activation was delayed. This suggests that these mutants, because they are unable to activate the Goq/11-PLC pathway, promote ERK1/2 phosphorylation by transactivating EGFR. This is supported by the fact that AG1478 entirely abolished UII-induced ERK1/2 phosphorylation in cells expressing the R1483.50A and R1483.50H mutants (Fig. 4, C and D). To confirm that the Arg1483.50 mutants were able to promote EGFR transactivation, COS-7 cells transiently transfected with wild-type UT receptor or Arg1483.50 mutant constructs were stimulated with UII and lysed. Presence of active tyrosine-phosphorylated EGFR was then confirmed with anti-phosphotyrosine antibodies (anti-p-Tyr). Figure 4E shows that activated UT receptor, as well as both Arg1483.50 mutants, promoted the tyrosine phosphorylation of EGFR. Moreover, this transactivation was delayed in cells expressing the Arg1483.50 mutants (Fig. 4, E and F). These results suggest that Arg1483.50 of the ERY motif is essential for the activation of Goq/11 but is not involved in the transactivation of EGFR and that the UT receptor activates two independent mechanisms that lead to ERK1/2 phosphorylation.

Role of Asp972.50, Glu1473.49, Arg1483.50, and Tyr1493.51 in Agonist-Dependent Cell Surface Receptor Loss. We showed previously that UT undergoes agonist-induced internalization in COS-7 cells (Proulx et al., 2005). Figure 5 shows that a 2-hour treatment with 0.1 μM UII caused a significant 20% loss of cell surface receptors on cells expressing the UT receptor. Under the same conditions, the D972.50A and both Arg1483.50 mutant receptors showed no significant loss of cell surface receptors, whereas the Y1493.51A mutant behaved as wild-type receptor. It is noteworthy that the agonist-induced cell surface loss of E1493.49A receptors increased considerably compared with wild-type receptor. To determine whether this was modulated by arrestins, we coexpressed β-arrestin2-GFP with the wild-type and mutant receptors. As shown in Fig. 5 (black columns), coexpression of β-arrestin2-GFP increased cell surface...
face receptor loss of wild-type, E1473.49A, and Y1493.51A mutant receptors by 100, 54, and 102%, respectively, but did not significantly affect the cell surface loss of the D972.50A or Arg1483.50 mutants.

Asp972.50 and Arg1483.50 Mutants Do Not Promote β-Arrestin Translocation. We reported previously that activated UT receptors traffic into intracellular vesicles in HEK-293 cells and colocalize with either β-arrestin1-YFP or β-arrestin2-GFP (Proulx et al., 2005). To determine whether activated mutant receptors were able to promote β-arrestin translocation, we coexpressed myc-tagged wild-type and mutant receptors with functional conjugated

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**Fig. 3.** Effect of UT receptor mutations on MAPK activation. COS-7 cells transiently transfected with either wild-type or mutant receptors were serum-starved overnight. They were then treated with 0.1 μM UII at 37°C for 0, 2, or 30 min in DMEM and lysed. Equal amounts of protein were resolved by SDS/PAGE. A, activated MAPK was identified by immunoblotting using phospho-MAPK specific antibody, which labeled the 44- and 42-kDa bands (ERK1 and ERK2, respectively). The total amount of MAPK was the same in all samples based on parallel blots probed with antibody directed against total MAPK. B, p-ERK1/2 bands were analyzed by densitometry and presented as -fold phosphorylation (mean ± S.E.M. n = 3) over UT basal. Statistical significance of the difference was assessed using one-way ANOVA and post hoc Dunnett test. # represents statistically significant differences (p < 0.05) of basal phosphorylation of mutants compared to basal phosphorylation of wild-type UT.

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**Fig. 4.** Arg1483.50 mutants promote ERK1/2 activation by transactivation of EGFR. COS-7 cells transiently transfected with wild-type receptor (A) or Arg148 mutants (C) were serum-starved overnight. They were then treated with 1 μM BIM and/or 0.5 μM AG1478 for 30 min, stimulated with 0.1 μM UII at 37°C for the indicated time in DMEM, and lysed. Equal amounts of protein were resolved by SDS/PAGE. Activated MAPK was identified by immunoblotting using phospho-MAPK-specific antibody, which labeled the 44- and 42-kDa bands (ERK1 and ERK2, respectively). The total amount of MAPK was the same in all samples based on parallel blots probed with antibody directly against total MAPK. B and D, p-ERK1/2 bands were analyzed by densitometry and presented as -fold phosphorylation (mean ± S.E.M. n = 3) over basal as reference. E, COS-7 cells transiently transfected with wild-type receptor, or Arg1483.50 mutants were serum-starved overnight. They were then stimulated with 0.1 μM UII at 37°C for the indicated time in DMEM and lysed. The EGFR was immunoprecipitated from cell extracts and Western blotted using antibodies for phosphotyrosine and total EGFR protein. F, p-Tyr-EGFR bands were analyzed by densitometry and presented as -fold phosphorylation (mean ± S.E.M. n = 3) over basal. Statistical significance of the difference was assessed using one-way ANOVA and post hoc Dunnett test. #, p < 0.05; **, p < 0.01; ***, p < 0.001.
β-arrestin2-GFP in HEK-293 cells. Under control conditions, UT was localized to the plasma membrane (Fig. 6A), whereas β-arrestin2-GFP was uniformly distributed in the cell cytosol. After exposure to agonist (30 min), both UT and β-arrestin2-GFP redistributed to vesicular structures of the cell, and UT colocalized with β-arrestin2-GFP (Fig. 6A, merged images). Under the same experimental conditions, D972.50A and Arg1483.50 mutants were also distributed in the plasma membrane under basal conditions, but exposure to UII modified neither their distribution nor the cytosolic distribution of β-arrestin2-GFP (Fig. 6B). However, E1473.49A and Y1493.51A behaved like the wild-type receptor distributing at the plasma membrane under control conditions and colocalizing with β-arrestin2-GFP in vesicular structure after exposure to UII.

Discussion

The aim of this study was to determine the role of the conserved residues/motif Asp2.50 and ERY on the functionality of the UT receptor. Five mutants were constructed, all of which displayed cell-surface expression levels and binding properties toward the natural ligand, UII, that were similar to that of the wild-type receptor, which suggests that the mutations did not significantly perturb the overall structure of the receptor.

Substitution of Asp2.50 in TMD2 by alanine in the UT receptor (D972.50A) led to a receptor that could no longer activate phospholipase C with ensuing lack of inositol phosphate production (Table 2). UII binding neither promoted loss of cell surface of the D972.50A receptor nor activated the MAPK pathway. Asp2.50 played a major role in the activation of class A GPCRs because mutations of this residue alter the signaling capacity of many GPCRs (Neve et al., 1991; Bihoraeu et al., 1993). An interaction between Asp2.50 and the conserved Asn7.49 of the NPxxY motif in TMD7 may be important in the activation mechanism of GPCRs (Sealfon et al., 1995; Urizar et al., 2005). Asn7.49 acts as an on/off switch by adopting different orientations in the inactive and active states (Govaerts et al., 2001; Urizar et al., 2005). In the inactive state, Asn7.49 is restrained toward TMD6, whereas in the active state, it probably adopts an orientation favoring an interaction with Asp2.50 (Urizar et al., 2005). Altering Asp7.49 in the UT receptor would abrogate an obligatory interaction with Asn7.49 in TMD7 needed to maintain the active state. Like other class A GPCRs, substituting the Asp2.50 residue in the UT receptor with another amino acid would profoundly affect the ability of the receptor to transit from an inactive to an active state upon agonist binding.

In many GPCRs, including rhodopsin, mutating Glu or Asp in the E/D3.49RY motif to either Ala or Asn might eliminate an ionic interaction with Arg7.50 and increase their constitutive activity (Mhaoût-Kodja et al., 1999; Alewijnse et al., 2000; Ballesteros et al., 2001). However, for some GPCRs, E/D3.49 substitutions do not induce constitutive activation, suggesting that this residue has other roles (Ohyama et al., 2002; Capra et al., 2004). Using inositol phosphate production as a functional assay, replacing Glu1473.49 with alanine (E1473.49A) in the UT receptor did not promote constitutive activation. Thus, a putative ionic interaction between Glu1473.49 and Arg1483.50 would not constitute a major determinant in maintaining basal state of UT.

The E1473.49A mutant exhibited two particular properties. First, activating this mutant did not produce a maximal response compared with the wild-type receptor and, second, the agonist-dependent cell surface loss was greater than the wild-type receptor. It is thus possible that the reduced signaling efficacy of E1473.49A may be due to structural changes in the activated receptor that influence Goαi1 protein coupling and/or that enable increased coupling to the internalization machinery thereby increasing its desensitization relative to the wild-type receptor.

As mentioned above, we observed that, after agonist binding, loss at the cell surface of the E1473.49A mutant was greater compared with the wild-type receptor, a process that was improved by overexpressing β-arrestin2 (similar results were obtained with β-arrestin1; data not shown), which points to a β-arrestin-dependent internalization mechanism. Marion et al. recently proposed a model whereby the proximal 10 residues of second intracellular loops, including the E/DRY motif in rhodopsin-like receptors, provide binding determinants for β-arrestin recognition (Marion et al., 2006). The highly conserved amino acids proline and alanine, which reside six residues at the C terminus of the E/DRY motif in rhodopsin-like receptors, provide binding determinants for β-arrestin recognition (Marion et al., 2006). The highly conserved amino acids proline and alanine, which reside six residues at the C terminus of the E/DRY motif, regulate β-arrestin binding and β-arrestin-mediated internalization. It has been suggested that a conformational change directed by the second intracellular loop, probably using the loop itself as a binding patch, functions as a switch for transitioning β-arrestin from its inactive form to its active receptor-binding state. Replacing Glu1473.49 in the UT receptor may thus influence the orientation of residues of the second intracellular loop, which is involved in recognizing β-arrestins and modulating the affinity of the activated receptor to β-arrestins. Our results thus suggest that residue Glu1473.49 plays an important role in the contribution of the second intracellular loop of the UT receptor to the internalization of the receptor. However, because our method using ELISAs reveals loss of cell-surface receptors after agonist stimulation, we cannot exclude the possibility that trafficking, degradation, and recycling processes may affect the overall cell surface expression of the Glu1473.49 mutant.

An analysis of the activation profile of the MAPK pathway using the wild-type UT receptor reveals an early PKC-dependent pathway and an EGFR-dependent pathway that are involved in the activation of ERK1/2 in COS-7 cells. These
two pathways probably mediate the signaling profile of the UT receptor toward ERK1/2 activation, because inhibiting PKC and EGFR abolished ERK1/2 activation altogether. Substituting the conserved residue Arg1483.50 by alanine or histidine completely abolished the ability of the UT receptor to promote Goq11 activation/inositol phosphate accumulation and abrogated the internalization of the receptor in COS-7 cells after UII binding. In HEK-293 cells, the Arg1483.50 mutants did not promote β-arrestin2-GFP translocation or colocalization to UII-bound receptors. However, UII binding to R1483.50A and R1483.50H mutants induced ERK1/2 activation at later (30 min) but not earlier (2 min) time points. This activation was abolished by the addition of AG1478, an EGFR kinase inhibitor. Moreover, stimulated Arg1483.50 mutants promoted EGFR activation as demonstrated in Fig. 4, E and F. This suggests that the R1483.50A and R1483.50H mutants, which are unable to promote the phospholipase C/inositol phosphate/PKC activation cascade, can still activate ERK1/2 in a Goq11 protein-independent manner by transactivation of EGFR. ERK1/2 activation by GPCRs involves a complex network of G protein-dependent and -independent signals (Luttrell, 2003). For example, the angiotensin II type 1A (AT1) receptor in HEK-293 cells activates ERK1/2 by distinct pathways, either G protein or β-arrestin dependent (Ahn et al., 2004). Moreover, Goq11-independent signaling via EGFR transactivation as the main transductional mechanism for ERK1/2 activation has also been described for AT1 in CHO-K1 and in human coronary artery smooth muscle cells (Miura et al., 2004). In the case of the UT receptor, the fact that the Arg1483.50 mutants maintained their ability to activate ERK1/2 but were unable to recruit β-arrestin suggests that ERK1/2 activation is β-arrestin-independent.

Mutations in the DRY motif of other GPCRs such as the angiotensin II AT1 receptor overexpressed in the hearts of transgenic mice cause cardiac hypertrophy (Zhai et al., 2005), lending weight to the importance of this motif in GPCR function. In addition, an Arg3.50-to-His mutation in the vasopressin V2 receptor (V2R) has been shown to lead to a constitutively phosphorylated receptor (R137HV2R) that associates with β-arrestins and is sequestered in intracellular vesicles, thus behaving as a constitutively desensitized receptor (Barak et al., 2001). This mutated receptor has been associated with nephrogenic diabetes insipidus (Bichet, 1998), R137HV2R acting as a loss-of-function receptor (Barak et al., 2001). These observations, along with our results on the R1483.50H receptor, are of particular interest in light of a reported single nucleotide polymorphism (refSNP ID rs13305975) replacing Arg3.50 with His. Whether this polymorphism is linked to or could be a marker of a specific pathology remains to be addressed.

The urotensin II system has been implicated in the development of pathophysiological conditions such as cardiac hypertrophy (Tzanidis et al., 2003), which involves EGFR transactivation in cardiomyocytes (Onan et al., 2004). Whether UT receptor activation in a normal or mutated genetic background promotes cardiac hypertrophy via a...
...invariantly exert strongly disruptive effects on receptor activity and decrease agonist affinity, indicating that Arg \(^{50}\) is involved in receptor-G protein-coupling/activation rather than constrain the receptor in its ground state. An in-depth analysis of the ERY motif of the UT receptor will be needed to...


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