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Mutational Analysis of the Highly Conserved ERY Motif of the Thromboxane A_2 Receptor: Alternative Role in G Protein-Coupled Receptor Signaling

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ABSTRACT

The presence of highly conserved amino acid stretches in G protein-coupled receptors (GPCRs) usually predicts an important role in receptor function. Considerable attention has therefore been focused on the involvement of the highly conserved Glu/Asp-Arg-Tyr (E/DRY) motif at the cytoplasmic end of transmembrane domain 3 in the regulation of GPCR conformational states and/or the mediation of G protein activation. In the present study, we investigated the role of Glu^{129} and Arg^{130} in the ERY of thromboxane A_2 receptor α (TPα) in transfected human embryonic kidney 293 cells. We show that no conservative or nonconservative substitutions of Glu^{129} and Arg^{130} generated a constitutively active TPα mutant, but a nonconservative mutation of Arg^{130} (R130V) yielded a mutant receptor with significantly impaired 9,11-dideoxy-9α,11α-methanoepoxy-prosta-5*Z*,13E-dien-1-oic acid (U46619)-induced accumulation of inositol phosphates (IPs). This loss-of-function phenotype seems to be caused by the uncoupling of the TPα receptor from G_q, as demonstrated by the loss of high-affinity agonist binding, and not by receptor internalization, as shown by localization studies with the R130V-green fluorescent protein fusion protein. It is interesting to note that U46619-induced activation of the nonconservative E129V mutant stimulated the production of IPs with a ~10-fold lower EC_{50} and a ~2-fold higher E_{max} than in the wild-type receptor. Collectively, these data demonstrate that, unlike other GPCRs, mutations of Glu^{129} do not induce constitutive activity, whereas Arg^{130} is involved in G protein coupling or recognition, and they suggest the existence within class A GPCRs of at least two different subclasses that make different uses of the highly conserved E/DRY motif.

Thromboxane A_2, a by-product of the oxidative metabolism of arachidonic acid, is a potent stimulator of platelet activation and a constrictor of vascular and airway smooth muscle cells. In humans, it exerts its action by interacting with two splice variants (α and β) of a single G protein-coupled receptor (GPCR), termed TP (Coleman et al., 1995), which was originally cloned from a human placenta cDNA library (Hirata et al., 1991). The TP receptor has been shown to couple to different G proteins, mainly of the $G_{q/11}$ subfamily, causing the activation of phospholipase C and the breakdown of phosphatidylinositol in a variety of cell types (Shenker et al., 1991; Knezevic et al., 1993; Kinsella et al., 1997).

GPCRs represent the largest group of receptors for neurotransmitters and hormones, with more than 800 putative members having been identified in the human genome (Fredriksson et al., 2003). Although all GPCRs share a similar topology, sequence analysis does not predict common

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ABBREVIATIONS: GPCR, G protein-coupled receptor; TPα, thromboxane A_2 receptor α; ETC, extended ternary complex model; SQ29,548, [1*S*-[1α,2α(*Z*),3α,4α]]-7-[3-[[2-[(phenylamino)carbonyl]hydrazino]methyl]-7-oxabicyclo-[2.2.1]hept-2-yl]-5-heptenoic acid; I-BOP, [1*S*-[1α,2α(*Z*),3β(1*E*,3*S**),4α]]-7-[3-[3-hydroxy-4-(4-iodophenoxy)-1-butenyl]-7-oxabicyclo[2.2.1]hept-2-yl]-5-heptenoic acid; U46619, 9,11-dideoxy-9α,11α-methanoepoxy-prosta-5*Z*,13*E*-dien-1-oic acid; GFP, green fluorescent protein; HEK, human embryonic kidney; IP, inositol phosphate; V_2R, vasopressin type II receptor; α_{2A}-AR, α_{2A}-adrenergic receptor; AChR, acetylcholine receptor; WT, wild type; PCR, polymerase chain reaction; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum.

binding interface(s) between receptor and G proteins or consequent receptor functions (Bourne, 1997; Bockaert and Pin, 1999; Wong, 2003). Thus, the molecular mechanism of receptor-G protein interaction and activation remains poorly defined, especially concerning TP receptor activation and G protein contact sites (Hirata et al., 1994; D'Angelo et al., 1996; Zhou et al., 1999).

Considerable attention has been focused on the role of Glu/Asp-Arg-Tyr triplet of residues (the so-called "E/DRY motif"), located at the boundary between transmembrane domain 3 and the second intracellular loop of class A GPCRs (rhodopsin family), which includes the TP receptor (α group, prostaglandin receptor cluster) (Fredriksson et al., 2003). This highly conserved motif has been implicated in the regulation of receptor conformational states and/or in the mediation of G protein activation in a number of different GPCRs (Acharya and Karnik, 1996; Shibata et al., 1996; Lu et al., 1997; Scheer et al., 1997, 2000; Alewijnse et al., 2000; Chung et al., 2002).

Indeed, nonconservative mutations of the Arg residue in the DRY motif of a number of GPCRs, such as rhodopsin, adrenergic, histamine, and muscarinic receptors, have been characterized as displaying a "loss-of-function phenotype" (Franke et al., 1992; Zhu et al., 1994; Burstein et al., 1998; Alewijnse et al., 2000; Scheer et al., 2000; Chung et al., 2002). However, for most of these receptor mutants, agonist affinity seems to be either increased or unchanged, which suggests, according to the extended ternary complex (ETC) model, a conserved coupling with cognate G protein (Samama et al., 1993). A clear explanation for this apparent paradox has not been proposed so far. Very recently, it was suggested that mutations in the highly conserved Arg of the vasopressin type II receptor (V_2R) produce a "constitutively desensitized phenotype", reported as a loss-of-function mutant that displays decreased expression at the plasma membrane (Barak et al., 2001). These data have been successively extended to other GPCRs, suggesting that this emerging paradigm of constitutive receptor desensitization might represent a general mechanism of hormonal resistance (Wilbanks et al., 2002).

Nevertheless, in a small subgroup of receptors, including the α_{2A}-adrenergic receptor (α_{2A}-AR), the muscarinic m1 (m1 AchR), and possibly m5 AchR, nonconservative mutations of the central Arg of the E/DRY motif, besides impairing signal transduction, actually decrease agonist affinity (Burstein et al., 1998; Zhu et al., 1994; Chung et al., 2002), this time in accordance with the ETC model. Furthermore, in this latter group of receptors, mutations of the Glu/Asp residues did not induce constitutive activity (Lu et al., 1997; Burstein et al., 1998; Chung et al., 2002), as was the case for other class A GPCRs such as rhodopsin, α_{1B}-AR, V_2R, β_2-AR, and histamine H_2 receptors (Acharya and Karnik, 1996; Scheer et al., 1997; Morin et al., 1998; Rasmussen et al., 1999; Alewijnse et al., 2000). These observations have given rise to the hypothesis that the E/DRY motif may not have the same function in all class A GPCRs (Burstein et al., 1998; Chung et al., 2002).

Here, we investigated how Glu and Arg substitutions in the ERY motif of the TPα receptor affect its binding, signaling, and G protein interaction. In addition, we determined their subcellular localization, using confocal microscopy imaging of the chimeric wild-type (WT) and mutant TPα-green fluorescent protein (GFP) receptors. Our findings indicate that TPα receptor is resistant to constitutive activation when mutated in its ERY motif, which plays a fundamental role in G protein recognition. This suggests a function for this motif different from that commonly accepted for other GPCRs in regulating TPα receptor signaling.

Materials and Methods

Materials. cDNA for TPα was kindly provided by Dr. Colin Funk (University of Pennsylvania, Philadelphia, PA). Transformer Site-Directed Mutagenesis Kit, pEGFP-N2 vector, Advantage 2 PCR Kit, and BMH 71-18 mutS competent cells were from BD Biosciences Clontech (Palo Alto, CA). Epicurian Coli XL-1Blue competent cells were from Stratagene (La Jolla, CA). Restriction enzymes and molecular weight markers were purchased from New England BioLabs (Beverly, MA) and MBI Fermentas (Vilnius, Lithuania). Oligonucleotides were synthesized and sequenced by MWG Biotech (Ebersberg, Germany). QIAprep Spin Miniprep Kit and QIAfilter Plasmid Midi Kit were purchased from QIAGEN GmbH (Hilden, Germany). Transfection reagent ExGen 500 was from MBI Fermentas, and FuGENE 6 was from Roche Diagnostics (Indianapolis, IN). Cell-culture media, serum, supplements, and molecular biology reagents were purchased from Invitrogen (Carlsbad, CA). Inositol-free Dulbecco's modified Eagle's medium (DMEM) was from ICN Pharmaceuticals Inc. (Costa Mesa, CA). HEK293 cells were obtained from American Type Culture Collection (Manassas, VA). Ultima Gold was from PerkinElmer Life and Analytical Sciences (Boston, MA), as were [5,6-^{3}H]SQ29,548 and myo-[2-^{3}H]inositol. SQ29,548, [1*S*-[1α,2α(*Z*),3β(1*E*,3*S**),4α]]-7-[3-[3-hydroxy-4-(4-iodophenoxy)-1-butenyl]-7-oxabicyclo[2.2.1]hept-2-yl]-5-heptenoic acid (I-BOP), and U46619 were from Cayman Chemical (Ann Arbor, MI) and were stored as stock solutions at −20°C. Anion exchange resin AG 1X-8 (formate form, 200–400 mesh) and Poly-Prep columns, and Lowry dye-binding protein reagents were from Bio-Rad (Hercules, CA). Fura 2/acetoxymethyl ester and Pluronic F-127 were purchased from Molecular Probes (Eugene, OR). All electrophoresis reagents were from J. T. Baker (Phillipsburg, NJ). All other reagents were of the highest purity available from Sigma-Aldrich (St. Louis, MO).

Construction of Mutant TPα Receptors. Specific base substitutions were introduced into the cDNA for TPα receptor using the Transformer Site-Directed Mutagenesis Kit according to the manufacturer's instructions. Where possible, the mutagenic primer also included a silent mutation to introduce a specific restriction site, and the putative mutant plasmids were screened before sequencing by digestion with the appropriate restriction enzyme. Mutant oligonucleotides were as follows: R130V, 5′-ATGGCCTCAGAGGTCTACCTGGGTATC-3′; R130E, 5′-GGCCTCAGAGGAGTACTTGGGTATCACCCGG-3′; R130K, 5′-GCCATGGCCTCAGAGAAGTACCTGGGTATC-3′; E129D, 5′-GGGGCCATGGCCTCCGATCGCTACCTGGG-3′; E129K, 5′-GGGGCCATGGCTTCGAAGCGCTACCTGGG-3′; and E129V, 5′-GCCGCCATGGCCTCCGTACGCTACCTGGG-3′. Plasmid DNA was purified with the QIAprep Spin Miniprep Kit and sequenced. Ultrapure plasmids for cell transfections were obtained with the QIAfilter Plasmid Midi Kit.

Construction of TPα-GFP Fusion Proteins. The WT TPα and R130V mutant receptors were inserted in frame into the pEGFP-N2 vector. In brief, the TPα receptor cDNAs cloned in the pcDNA3 vector were amplified by PCR using the oligonucleotide primers 5′-GCCAGTGTGCTGGAATTCGCG-3′ (upper primer, containing the EcoRI site, underlined) and 5′-ATAGGATCC**CCT**GCAGCCCGGA-3′ (lower primer, containing the BamHI site, underlined, and replacing the stop codon, in bold), and the Advantage 2 PCR kit. The resulting amplification products were digested using EcoRI and BamHI restriction enzymes and inserted into the purified expression vector (pEGFP-N2) that had been opened between the EcoRI/BamHI polylinker restriction sites using the respective enzymes. The resulting constructs were grown in competent *Escherichia coli*, isolated, and verified by sequencing.

Culture and Transfection of HEK293 Cells. HEK293 cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 50 U/ml penicillin, 100 μg/ml streptomycin, and 20 mM HEPES buffer, pH 7.4, at 37°C in a humidified atmosphere of 95% air and 5% CO_2. HEK293 cells were plated out to a convenient dilution to obtain a 50 to 60% confluence on the day of transfection into 12-well (total inositol phosphate formation assay) or 24-well (binding assay) tissue culture dishes previously coated with 0.2% gelatin, or 12-mm diameter glass coverslips (Ca^{2+} measurement). Transfection with WT or mutant constructs or with vector alone was performed by means of a mixture of linear polyethylenimine, ExGen 500, according to the manufacturer's instructions. In brief, HEK293 cells were transfected in DMEM plus 3% FBS with an optimized 3:1 ExGen 500/DNA ratio. Three hours after transfection, the medium was replaced with DMEM supplemented with 10% FBS. Equal protein content was ensured at the end of each assay by the Lowry dye-binding procedure.

Ligand Binding Assays. Receptor expression was monitored 48 h after the transfection. A mixed-type protocol was performed as described previously (Capra et al., 1998; Rovati, 1998) on confluent adherent cells in 250 μΛ of serum-free DMEM containing 0.2% (w/v) bovine serum albumin in the presence of 0.1 to 3 nM concentrations of the specific receptor antagonist [^{3}H]SQ29,548 (48 Ci/mmol), 0.01 to 10 μM concentrations of the homologous unlabeled ligand, or 0.001 nM to 1 μM concentrations of the heterologous unlabeled ligand I-BOP. After 30 min of incubation at 25°C, cells were washed with ice-cold phosphate-buffered saline containing 0.2% (w/v) bovine serum albumin and lysed in 0.5 N NaOH. Radioactivity was measured by liquid scintillation counting. Binding data were analyzed by means of the LIGAND program (Munson and Rodbard, 1980). All of the curves shown were generated by computer fitting.

Total Inositol Phosphate Determination. The functional activity of the receptor was assessed 48 h after transfection by measurement of the total inositol phosphate (IP) accumulation (Seuwen et al., 1988; Habib et al., 1997). In brief, confluent cells were labeled with 1 μCi of [*myo*-2-^{3}H]inositol (17 Ci/mmol) for 24 h in serum-free, inositol-free DMEM containing 20 mM HEPES buffer, pH 7.4, and 0.5% (w/v) Albumax I. Cells were washed and incubated with serum-free, inositol-free DMEM containing 25 mM LiCl for 10 min and then incubated with or without 1 μM U46619. After 30 min, the reaction was stopped by aspiration of the supernatant and the addition of 0.75 ml of 10 mM formic acid. After 30 min of incubation at room temperature, the solution was collected in 3 ml of 5 mM NH_4OH, pH 8 to 9, and separated with an anion exchange AG 1X-8 column, formate form, 200 to 400 mesh. Free inositol and glycerophosphoinositol were washed with 40 mM ammonium formate/formic acid buffer, pH 5, and total IP was eluted with 4 ml of a 2 M ammonium formate/formic acid buffer, pH 5, of which 250-ml aliquots were counted by liquid scintillation.

Measurements of the Concentration of Cytosolic Free Ca^{2+}. Ca^{2+} was measured by monitoring the intensity of Fura 2 fluorescence. Forty-eight hours after transfection, confluent cells were incubated for 45 min at 37°C in the dark with 5 μM Fura 2/acetoxymethyl ester in buffered salt solution (145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 10 mM HEPES, and 10 mM glucose, pH 7.4) plus 0.03% (v/v) Pluronic F-127. After loading, cells were washed twice with a saline solution. The coverslips were transferred to the spectrofluorimeter (PerkinElmer LS50) cuvette, and fluorescence was monitored at 37°C (505 nm emission, 340 and 380 nm excitation). To measure the concentration of cytosolic free Ca^{2+} ion, $[Ca^{2+}]_i$, from the fluorescence recording, the system was calibrated as follows: F_{max} (maximal fluorescence of the system) was obtained by adding 4 μM ionomycin and 100 μM digitonin, and F_{min} was obtained by adding 5 mM EGTA and 40 mM Tris base. $[Ca^{2+}]_i$ was calculated as described by Grynkiewicz et al. (1985) with a K_d value of 224 nM.

Confocal Microscopy. HEK293 cells were plated onto poly-L-lysine–coated glass coverslips in 35-mm dishes. After overnight incubation at 37°C, cells were transfected with 0.5 μg of WT TPα-GFP or R130V-GFP using the FuGENE 6 reagent (Roche). Two days after transfection, the cells were treated for different times with 1 μM U46619 at 37°C and fixed with 4% (w/v) paraformaldehyde in phosphate-buffered saline for 10 min at room temperature. Samples were viewed on a Bio-Rad Radiance 2000 laser-scanning confocal microscope using a Plan Apochromatic 60XA/1.40 numerical aperture oil objective under excitation by a 488-nm wavelength argon/krypton laser.

Statistical Analysis. Statistical analysis of ligand-binding data were performed with the LIGAND program (Munson and Rodbard, 1980), whereas concentration-response curves were analyzed with ALLFIT (De Lean et al., 1978). Models of increasing complexity were compared using the statistical principle of the "extra sum of squares" (Draper and Smith, 1966). Parameter errors are all expressed in percentage coefficient of variation (%CV) and calculated by simultaneous analysis of at least two different independent experiments performed in duplicate or triplicate. When indicated, analysis of variance followed by Bonferroni's post hoc test for multiple comparisons were performed. Data are presented as means ± S.E.M. of several independent experiments (at least three), with each performed at least in duplicate. A level of statistical significance of $p < 0.05$ was accepted.

Results

Whole-Cell Binding and Receptor Expression of TPα Mutants. Binding of the specific TPα antagonist [^{3}H]SQ29,548 was performed on normal or transiently transfected HEK293 cells with either the WT TPα receptor or the pcDNA3 vector alone ("mock"). Normal and mock-transfected cells showed no detectable binding in mixed-type curves of [^{3}H]SQ29,548 (data not shown), whereas cells transfected with the WT receptor displayed a monophasic binding curve fitting a single-site model by computer analysis (Fig. 2) with typical binding parameters (Table 1), as previously reported (Habib et al., 1997; Capra et al., 2003). Cells transfected with mutant receptors listed in Fig. 1 were also tested in binding studies. The substitution of Arg^{130} with Val or Lys and those of Glu^{129} with Val or Asp all resulted in receptors with binding profiles similar to that of the WT receptor (Fig. 2). LIGAND analysis of binding data showed that the affinities for the antagonist [^{3}H]SQ29,548 of the mutant receptors were not statistically different from those of the WT receptor (Table 1). Two of the mutants, R130E and E129K, were only negligibly expressed and could not be studied further.

Previous studies have indicated that some GPCRs with mutations at the Glu/Asp and Arg residues of the E/DRY motif showed a lower expression than the corresponding WT receptors (Lu et al., 1997; Scheer et al., 1997; Rasmussen et

TABLE 1
Binding affinities of [^{3}H]SQ29,548 in HEK293 cells transiently expressing the wild-type or the mutant human TPα receptors
Binding affinities and capacities were obtained by simultaneous analysis with the LIGAND program of several independent [^{3}H]SQ29,548 mixed-type experiments.

Receptor	K_d	B_{max}	No. of Experiments
	nM ± *%CV*	*pmol/mg protein* ± *%CV*	
Wild type	6.39 ± 12	0.54 ± 11	9
R130K	5.32 ± 10	0.67 ± 8	4
R130V	7.89 ± 24	0.69 ± 18	3
E129V	5.38 ± 9	0.46 ± 7	5
E129D	6.32 ± 16	0.45 ± 12	3
Wild type-GFP	8.96 ± 33	0.82 ± 23	2
R130V-GFP	10.38 ± 11	0.66 ± 8	2

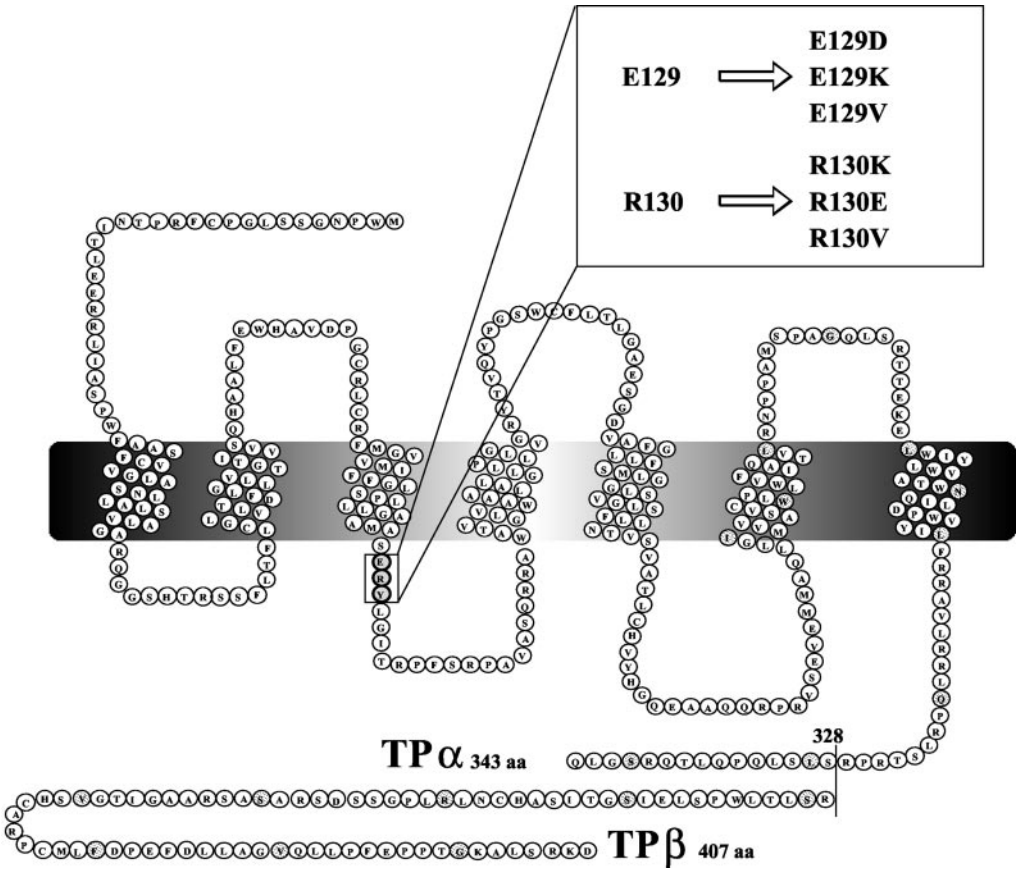

Fig. 1. Putative membrane topology of the TPα isoform receptor showing the position of the ERY motif at the N-terminal end of the second intracellular loop with point mutations described. The different cytoplasmic C-terminal ends of TPα and TPβ isoforms are shown. The blocked area represents plasma membrane with seven putative transmembrane domains.

al., 1999; Alewijnse et al., 2000; Chung et al., 2002). The same is true for the E129V-TPα mutant, whose level of expression in transfected HEK293 cells was much less than WT TPα ($\sim$10-fold reduction in [^{3}H]SQ29,548 binding sites when the same amount of DNA was used), whereas the level of expression of R130V, R130K, and E129D mutants was not statistically different from that of WT. To allow a proper comparison of receptor responses (see below), transfection conditions were therefore adjusted to secure equivalent levels of receptor expression for WT and all mutants (0.3–0.9 pmol/mg of protein).

Agonist Binding of TPα Receptor Mutants. WT and selected mutants transiently transfected in HEK293 cells were also tested for high-affinity agonist binding to assess whether the mutations affected G protein coupling or activation. Heterologous competition curves of the unlabeled agonist I-BOP versus [^{3}H]SQ29,548 for the WT receptor and conservative R130K and E129V mutants (Fig. 3) revealed the presence of a high-affinity binding component, and data were better fitted by a two-site model (Table 2). In contrast, competition curves generated for the R130V mutant spanned within 2 orders of magnitude (Fig. 3) and were better resolved by a single-site fit (Table 2), demonstrating the loss of the high-affinity component.

Signaling of TPα Receptor Mutants. We compared the functionality of WT and mutated TPα by measuring the agonist-induced total IP accumulation (Fig. 4). Basal IP levels of the TPα mutants were not significantly different from that with WT, indicating that none of the receptors displayed constitutive activity. HEK293 cells expressing the WT TPα responded to 1 μM U46619 stimulation with a marked ele-

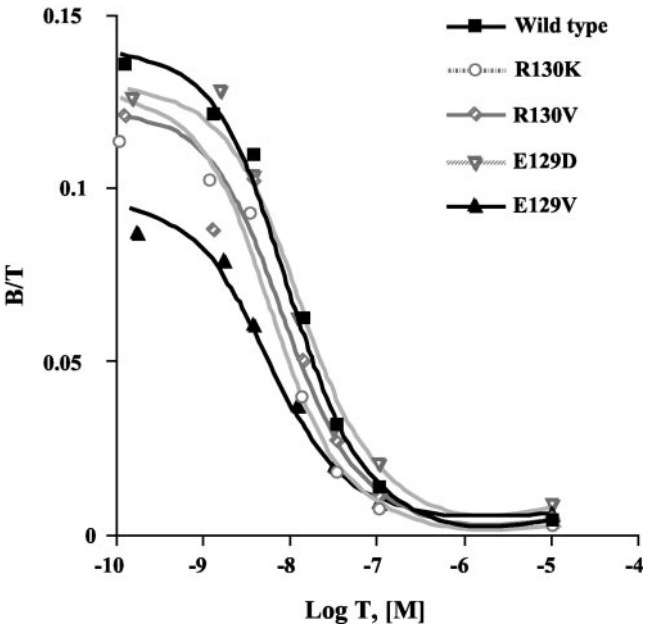

Fig. 2. Equilibrium mixed-type binding curves of TP antagonist [^{3}H]SQ29,548 in HEK293 cells transiently expressing the WT and mutant TPα receptors. Binding of [^{3}H]SQ29,548 is expressed as the ratio of bound ligand concentration to total ligand concentration (B/T, dimensionless) versus the logarithm of total ligand concentration (Log T). T is the sum of labeled and unlabeled ligand. Nonspecific binding was determined by computer analysis as one of the unknown parameters of the model and was always $<$10% of total binding. Several independent [^{3}H]SQ29,548 mixed-type experiments were performed, each with duplicate determinations, and were analyzed simultaneously (see Table 1). For the sake of clarity, only one representative curve for each receptor is shown. All curves are computer-generated.

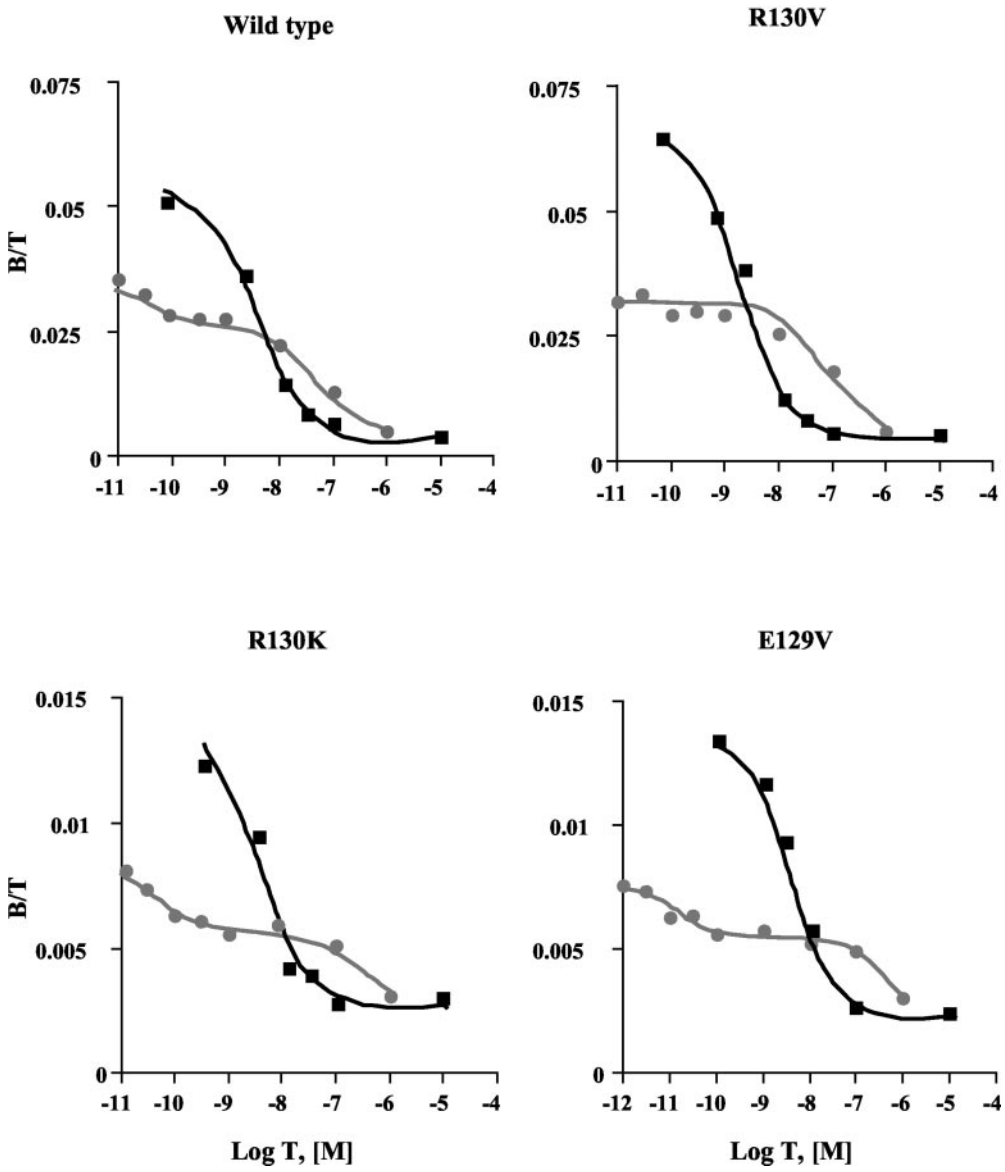

Fig. 3. Agonist binding studies in HEK293 transiently expressing WT and mutated TPα receptors. [^{3}H]SQ29,548 mixed type curves and I-BOP heterologous competition curves are shown for WT, R130V, R130K, and E129V mutant receptors. Binding is expressed as the ratio of bound ligand concentration to total ligand concentration (B/T, dimensionless) versus the logarithm of unlabeled ligand concentration (Log T). Nonspecific binding was calculated by computer analysis as one of the unknown parameters of the model and was always <10% of total binding. Several independent heterologous competition experiments were performed, each in duplicate, and were analyzed simultaneously (see Table 2). For the sake of clarity, only curves from one representative experiment for each receptor are shown. All curves are computer-generated, ■, SQ29,548; ●, I-BOP.

vation of total IPs (4.86 ± 0.52-fold increase over basal). In contrast, the 130V mutant responded poorly to agonist (1.87 ± 0.12- fold increase, 77% less than WT), whereas the two conservative R130K and E129D mutants were not statistically different from WT receptor (3.86 ± 0.17- and 4.70 ± 0.5-fold increases, respectively).

We also investigated the relationship between cellular levels of receptor expression and total IP formation for WT and R130V mutant receptors. Figure 5 shows that although a clear correlation exits between WT receptor expression and agonist-induced IP formation ($p < 0.01$), no such relationship exists between IP formation and R130V mutant expression or between basal IP levels and WT or R130V receptor expression.

Functional coupling of the WT and R130V mutant receptors was also assessed by monitoring Ca^{2+} transients in response to 1 μM U46619. Figure 6 shows that HEK293 cells transfected with the WT TPα responded to agonist with a transient increase in the concentration of $[Ca^{2+}]_i$, whereas agonist stimulation of cells expressing the R130V mutant elicited only a small calcium transient. Cumulative data from at least three independent experiments confirmed that agonist stimulation of the mutant receptors induced only approximately 30% of the $[Ca^{2+}]_i$ mobilization of WT receptor (Fig. 6B).

Unlike the other mutants, E129V produced a significant increase in agonist-induced total IP formation (8- to 9-fold over basal), again in the absence of any increase in constitu-

TABLE 2

Binding affinities of I-BOP vs. [^{3}H]SQ29,548 in HEK293 cells transiently expressing the wild-type or mutant TPα receptors

Binding affinities were obtained by simultaneous analysis with LIGAND of at least two independent homologous and heterologous competition curves. Models of increasing complexity (i.e. one-site vs. two-site model) were compared as described under *Materials and Methods,* and a statistical level of significance of $p < 0.05$ was accepted.

Receptor	K_{i1}	K_{i2}	B_{max1}/B_{max2}	No. of Experiments
	nM ± %CV	*nM ± %CV*		
Wild type	0.012 ± 75	38 ± 27	0.39	4
R130K	0.011 ± 78	143 ± 62	0.41	2
R130V		44 ± 23		4
E129V	0.007 ± 85	6.5 ± 15	0.54	3

tive activity (Fig. 4). To investigate the nature of this effect further, we constructed U46619 concentration-response curves for the WT and E129V mutant receptors (Fig. 7). ALLFIT-assisted analysis of total IP formation indicated a statistically significant ($p < 0.01$) 10-fold leftward shift in EC_{50} values between WT and the E129V mutant (EC_{50} = 150.6 nM ± 9.7% CV and 15.9 nM ± 5% CV, respectively) and approximately a 2-fold increase of the E_{max} value (E_{max} = 1799 dpm ± 1.9% CV and 3420 dpm ± 1% CV, respectively).

Cellular Distribution of TPα-GFP Fusion Chimeras. Finally, to check whether the impairment in signaling of the R130V mutant might be caused by constitutive internalization, we analyzed by confocal microscopy the subcellular distribution of the WT and R130V mutant TPα receptors tagged with GFP. Figure 8 illustrates that in HEK293 cells transfected with either WT or R130V GFP-tagged receptors, fluorescence was predominantly associated with the surface, which suggests a plasma-membrane localization in the steady state. The addition of 1 μM U46619 for up to 60 min did not modify the localization of the GFP-tagged WT and R130V receptors. The pharmacology (antagonist binding and total IP formation) of both chimeras was not statistically different from their respective untagged counterparts (Table 1 and Fig. 4).

Discussion

In this report, we used site-directed mutagenesis of the TPα receptor to study the role of the highly conserved ERY motif located at the cytosolic end of transmembrane helix 3. We have shown that none of the mutant receptors transiently expressed in HEK293 cells displayed a constitutive activity;

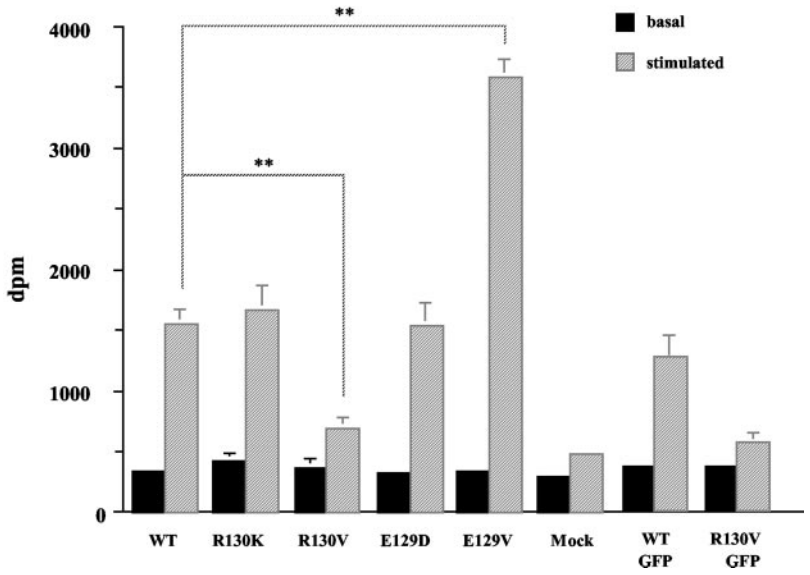

Fig. 4. Basal and agonist-induced total IP formation. A and B, all TPα receptors were transiently expressed in HEK293 cells at levels ranging from 0.3 to 0.9 pmol/mg of protein, and IP accumulation was measured after incubation in the absence (basal) or presence of 1 μM U46619 agonist (stimulated) for 30 min. Data are expressed as mean ± S.E. from three to seven independent experiments, each performed in triplicate. ⋆⋆, $p < 0.01$ versus agonist-stimulated IP accumulation of WT TPα (analysis of variance followed by Bonferroni post hoc test).

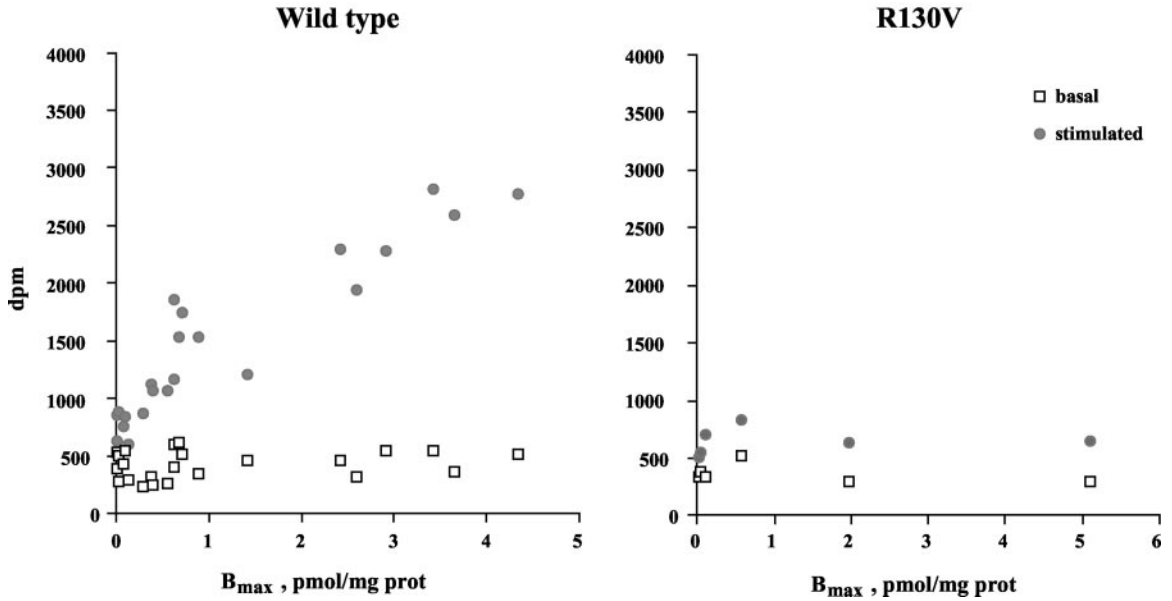

Fig. 5. Relationship between basal or agonist-induced total IP formation and level of expression of the WT or R130V TPα receptors. These were transiently expressed in HEK293 cells at levels up to 5 pmol/mg of protein, and IP accumulation was measured after incubation in the absence (basal) or presence of 1 μM U46619 (stimulated) for 30 min. Each data point (basal and stimulated) represents a single independent experiment performed at a different expression level of TPα receptor determined by [^{3}H]SQ29,548 binding.

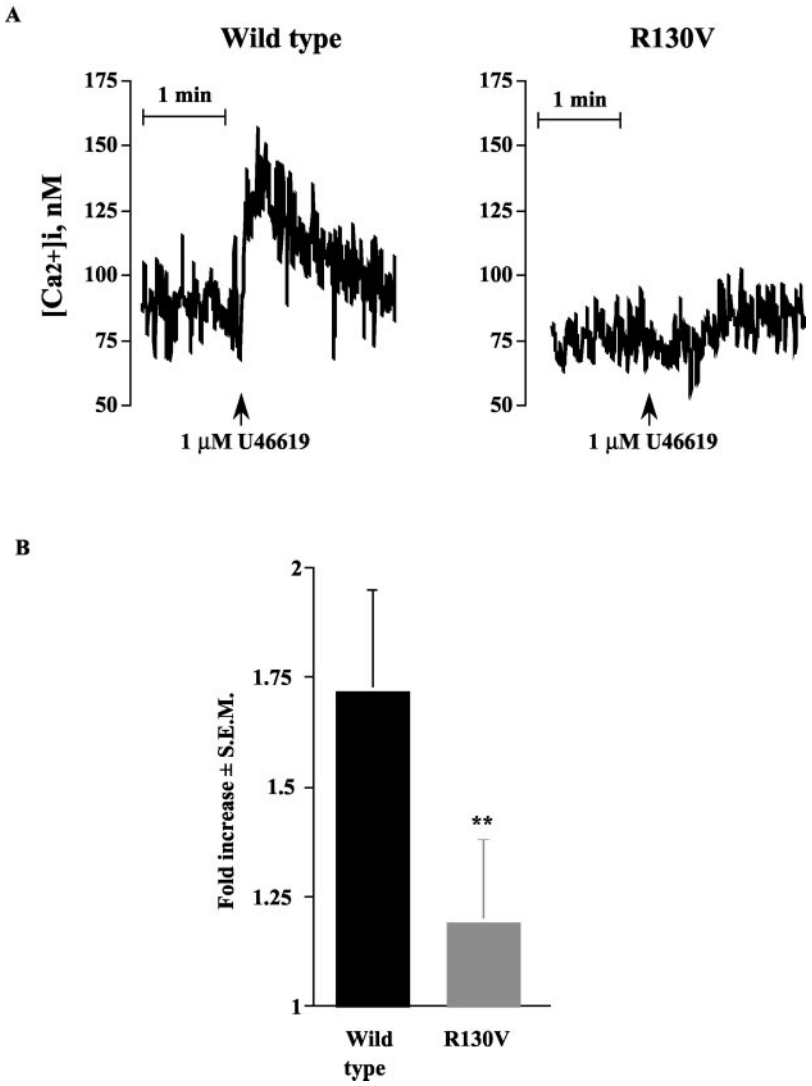

Fig. 6. Calcium measurements in HEK293 cells expressing WT and R130V receptors. U46619-induced increase in $[Ca^{2+}]_i$ was measured by monitoring the fluorescence of cells loaded with Fura 2. A, representative tracing of agonist-induced increase in $[Ca^{2+}]_i$ in HEK293 expressing the WT (left) and R130V mutant (right) receptors. B, increases (x-fold) of intracellular calcium induced by agonist stimulation of the WT and R130V mutant receptors. Data are expressed as means ± S.E. from four independent experiments each performed in triplicate. ⋆⋆, $p < 0.01$ versus WT receptor (independent t test).

rather, the nonconservative R130V mutation strongly impaired agonist-induced, $G_{q/11}$-dependent, total IP formation and $[Ca^{2+}]_i$ mobilization. This loss-of-function phenotype seems to be caused by the uncoupling of TPα receptor from the cognate G protein and not by reduced receptor presence at the plasma membrane surface, thus implying that Arg^{130} residue of the ERY motif is a key residue for G protein coupling. We also observed that, despite being less well-expressed than WT receptor, the nonconservative E129V mutant increased the accumulation of IPs more than did WT upon stimulation by the U46619 agonist. Thus, collectively, our findings suggest that the canonical model of the role of E/DRY function in GPCRs does not apply to the TPα receptor, but rather that alternative functions for this highly conserved motif exist within class A GPCRs.

Although the regions involved in G protein coupling in a specific GPCR subfamily are usually not well-conserved within other receptors coupled to the same G protein family, it is also clear that there are no consensus sequences for the binding of the α subunit of the G proteins to GPCRs (Bockaert and Pin, 1999; Wess, 1997, 1998; Gether, 2000). However, the presence of stretches of highly conserved residues among class A GPCRs (Fredriksson et al., 2003), such as the E/DRY motif, suggest an important role for these regions in receptor function. Very recently, work derived from evolutionary tracing has identified three clusters of residues commonly important in diverse GPCRs (Madabushi et al., 2004). Furthermore, it has been recognized that the hydropathy index of the residues involved in receptor-G protein interaction and/or activation is important for signal transduction (Moro et al., 1993; Scheer et al., 1997; Wess, 1998).

We therefore decided to mutate the Glu and Arg residues within the ERY motif of the TPα receptor by nonconservative (E129V or R130V) or conservative (E129D and R130K) substitutions. Our antagonist-binding data indicate that the WT and all mutant receptors heterologously expressed in HEK293 cells have similar K_d values in the nanomolar range. Although the other mutants did not show any appreciable variations in the total number of binding sites, the E129V mutant showed a significant reduction of expression (E129K and R130E were not expressed). This phenomenon has already been observed with other receptors mutated in the E/DRY motif such as the α_{1B}-AR (Scheer et al., 1996, 1997), α_{2A}-AR (Chung et al., 2002), H_2 (Alewijnse et al., 2000), and others. The reason for this decreased surface expression is not yet clear, but reduction in receptor stability, misfolding, or, more recently, receptor desensitization and internalization has been proposed (Wilbanks et al., 2002).

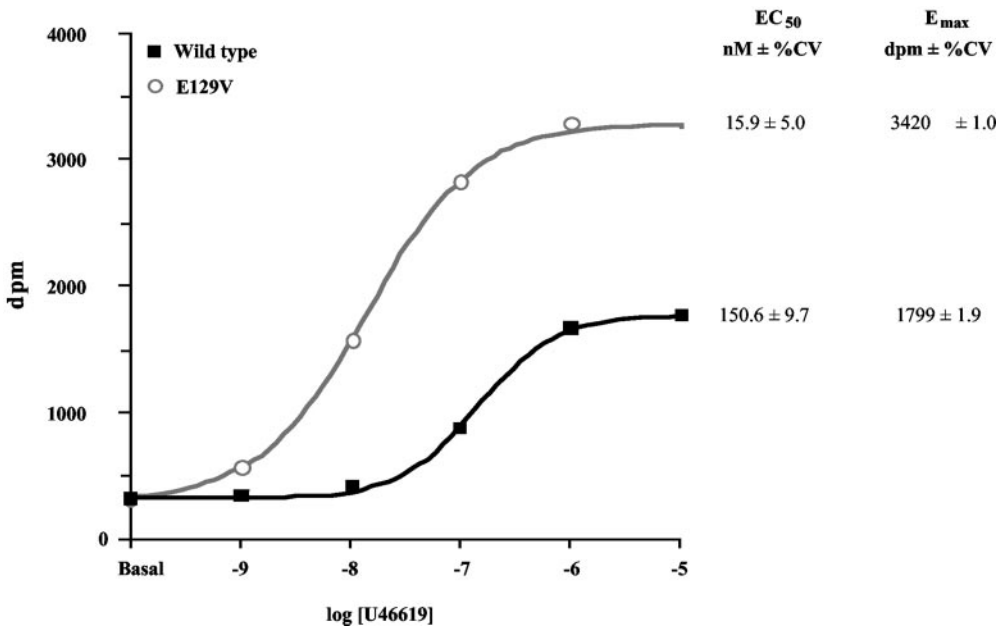

Fig. 7. Concentration-response curves of agonist-induced total IP formation in HEK293 cells expressing the WT and E129V receptors (0.30 and 0.35 pmol/mg of protein for WT and E129V, respectively). IP accumulation was measured after incubation in the absence (basal) or presence of increasing concentrations of U46619 agonist for 30 min. Values of EC_{50} and E_{max} were obtained by simultaneous analysis with the ALLFIT computer program of three different experiments performed in duplicate. Curves are computer-generated.

To allow a direct comparison of basal and agonist-induced IP accumulation, we adjusted transfection conditions so to have equal expression of the WT and all mutant receptors. No Arg mutations resulted in receptors with increased constitutive activity, but the nonconservative R130V mutation generated receptors with a statistically significant impairment in agonist-induced total IP production, demonstrating that this residue is indeed important for receptor functionality. The conservative mutation R130K did not affect receptor signaling. However, the exact role of Arg in the E/DRY motif is still under discussion. It has been suggested that Arg may catalyze GDP release (Acharya and Karnik, 1996) or may be involved in receptor isomerization (Scheer et al., 2000), but it is also possible that this residue is directly involved in G protein recognition and coupling (Burstein et al., 1998; Chung et al., 2002).

To examine this problem further, we performed heterologous displacement curves using the TP receptor agonist I-BOP as a competitor of the labeled antagonist [^{3}H]SQ29,548. It is well-known that, in the case of GPCRs, agonists can demonstrate high-affinity binding caused by the promotion of G protein coupling and ternary complex formation (De Lean et al., 1980). When performing heterologous displacement curves with the I-BOP agonist, we obtained biphasic curves characterized by the presence of a high- and a low-affinity component for the WT receptor and the conservative R130K mutant and low-affinity monophasic curves in the case of the R130V mutant receptor. Together with the lack of constitutive activity of any Arg mutant, these data imply that the loss of the high-affinity agonist binding and the loss of function of the R130V mutant are secondary to a defective G protein coupling. This might be ascribed either to disruption of the physical interaction between receptor and G protein or to a reduced receptor affinity for the cognate G protein.

It is thus becoming clear that the mutation of this key Arg produces at least two distinct phenotypes in class A GPCRs: the first is characterized by conserved high-affinity agonist binding and G protein coupling (α_{1B}-AR, β2-AR, H_2R, and V_2R), and the second is characterized with a loss of high-affinity agonist binding and, conceivably, a loss of G protein coupling (α_{2A}-AR, m1 AchR, possibly m5 AchR, and TPαR). These observations suggest a different role for this residue and probably for the entire E/DRY motif in regulating receptor function. It is interesting that both phenotypes are often characterized by a loss of function, sometimes explained by constitutive desensitization (Wilbanks et al., 2002). However, confocal imaging of GFP-tagged receptors demonstrated that neither the WT nor the R130V mutant undergoes agonist-induced internalization, ruling out the possibility that the loss-of-function phenotype is secondary to endocytosis. Indeed, these data, extending previous observations that TPβ but not TPα undergoes agonist-promoted internalization (Parent et al., 1999), confirm that, at least for TPα, receptor down-regulation is not responsible for functional impairment.

Unlike the Arg^{130} mutants, both mutants targeting the Glu^{129} residue retained their signaling ability, once again without any increase in basal receptor activity. Indeed, E129V had a number of effects that support an important role for Glu^{129} in stabilizing TPα conformation. The E129V mutant displayed a 2- to 6-fold increase in agonist affinity, in addition to a 10-fold reduction in receptor expression. This phenomenon has already been observed for α_{2A}-AR (Chung et al., 2002) and m1 AchR (Lu et al., 1997), and it has been interpreted as a possible conformational change in the agonist-binding pocket. Agonist stimulation of the E129V mutant doubled efficacy and increased agonist potency 10-fold over the WT receptor in IP formation. To our knowledge, E129V represents the first example of a mutation in the E/DRY motif that does not increase basal activity while augmenting agonist-stimulated receptor signaling. These results led us to hypothesize a conformational change of the receptor toward an active-like conformation, in accordance with the ETC model (Samama et al., 1993). However, differences in the affinities between the WT and the E129V mutant are lower than the limit of resolution of the binding experiments and thus do not allow for a definitive conclusion at this stage.

In conclusion, we propose that within class A GPCRs, at

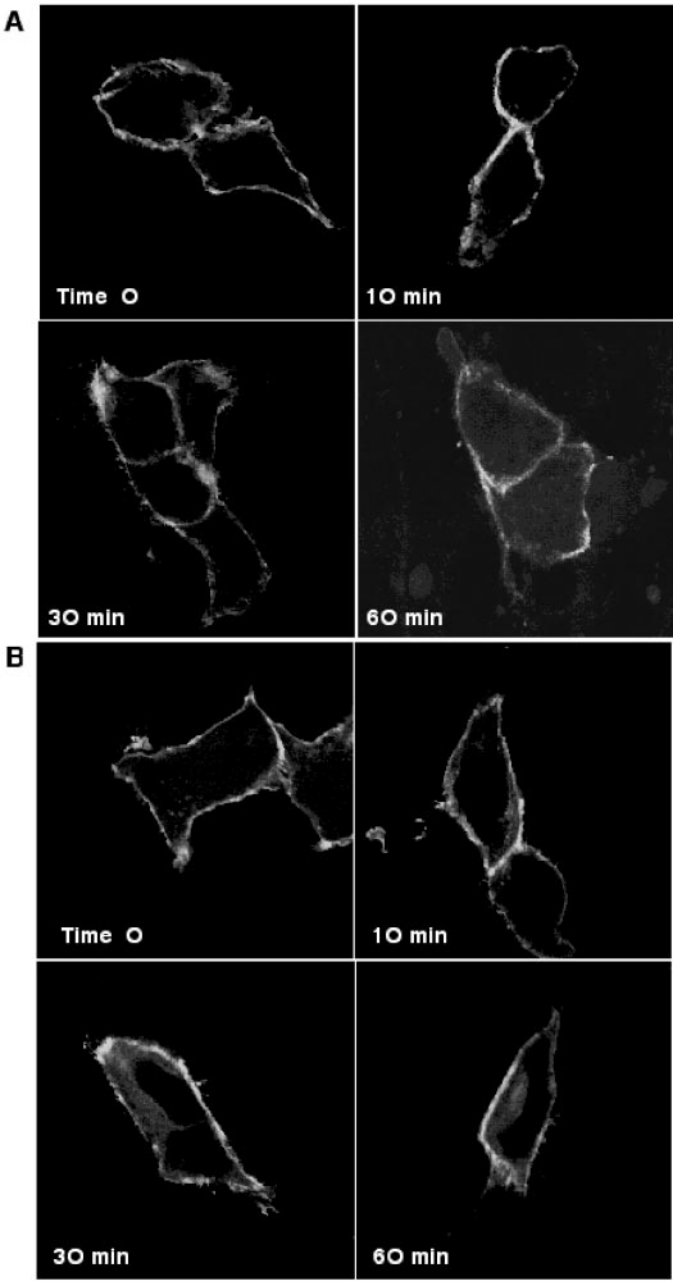

Fig. 8. Confocal microscopy imaging of GFP-tagged WT (A) and R130V mutant (B) in transiently transfected HEK293 cells. Cells were fixed with paraformaldehyde 48 h after transfection in basal conditions (time 0) and after stimulation with 1 μM U46619 at the indicated time points. One representative experiment is shown.

least two different subgroups of receptors exist that make different uses of the E/DRY motif independently of the class of G protein (G_s, G_i, or G_q) to which the receptor is preferentially coupled (Burstein et al., 1998; Chung et al., 2002). In the first group (α_{1B}-AR, β2-AR, H_2R, V_2R, and possibly other GPCRs) this highly conserved motif is involved in constraining the receptor in the ground state. Therefore, all nonconservative mutations of the Glu/Asp-Arg residues increase or induce constitutive activity of the receptors, increase (or not affect) affinity for agonist binding, and retain G protein coupling and an agonist-induced response that is sometimes evident but is sometimes masked by an increase in receptor internalization (constitutively desensitized receptor, apparent loss-of-function phenotype). In contrast, TPα receptor joins the α_{2A}-AR, m1 AchR, and probably the m5 AchR group of receptors in which the E/DRY motif is more directly involved in governing G protein coupling/recognition. Hence, mutations of the Glu/Asp residues do not induce constitutive activity, whereas agonist-induced responses might be altered in a mutation-specific manner. Indeed, some nonconservative mutants might yield receptors with more efficient signaling properties, an observation that seems to suggest a conformational change. On the other hand, the central Arg of the ERY motif seems to be more directly involved in receptor-G protein coupling/recognition so that nonconservative mutations of this residue invariably impair agonist-induced receptor responses and, accordingly, reduce affinity for agonist binding. This study also confirms the importance of the hydropathic characteristic of the residues involved in G protein-receptor binding, as suggested by others previously (Moro et al., 1993; Scheer et al., 1997; Wess, 1998). In fact, substitutions with residues having conserved hydropathic characteristic (E129D and R130K) had no effect in TPα receptor functionality.

It is likely that other subclasses may exist within class A GPCRs characterized by a different function of the conserved E/DRY motif. More data in an ever-increasing number of different receptors will clarify the role of this highly conserved triplet in GPCR activation and function.

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