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Substitution of Charged Amino Acid Residues in Transmembrane Regions 6 and 7 Affect Ligand Binding and Signal Transduction of the Prostaglandin EP_3 Receptor

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SUMMARY

Expression of the rabbit EP_3 receptor isoform 77A in COS1 and HEK293tsA201 cells demonstrated specific binding of [^{3}H]prostaglandin (PG)E_2 and receptor-evoked decreases in intracellular cAMP levels. Competition binding with PGE_2, PGE_2 methyl ester, misoprostol-free acid, misoprostol, and sulprostone suggested that a negative charge at the C1 position is essential for high affinity ligand binding and that the charge at this position is more important than steric bulk. Charged amino acid residues within the transmembrane (TM) domains of the receptor were mutated, and the resulting receptor proteins were analyzed for the effects of these mutations on receptor structure and/or function. Positively charged TM residues are candidates for interaction with the C1 carboxylic acid moiety of prostanoid ligands. Substitution of R329 (TM VII) with either alanine or glutamate resulted in a loss of both detectable [^{3}H]PGE_2 binding and receptor activation despite expression of the receptor protein as determined by immunoprecipitation and immunofluorescence. Substitution of K300 (TM VI) with alanine had no effect on binding or signal transduction. Substitution of the conserved aspartic acid at position 338 (TM VII) with alanine caused a loss of detectable receptor-evoked inhibition of cAMP generation, although this mutation did not appreciably affect ligand binding. These studies suggest that R329 but not K300 is a key determinant in receptor/ligand interaction. Furthermore, D338 plays a critical role in G_i activation by the EP_3 receptor.

PGE_2 is an arachidonic acid metabolite that mediates a broad range of physiological effects through interactions with specific cell surface receptors. Molecular cloning has identified at least four distinct E-prostanoid receptor subtypes, designated EP_1, EP_2, EP_3 and EP_4, which are members of the superfamily of seven-TM GPCRs. The EP_3 receptor subtype modulates water and ion transport in the kidney (1), gastric acid secretion (2), neurotransmitter release (3), hepatic glucose metabolism (4), and smooth muscle tone (5). Molecular biological strategies have revealed multiple EP_3 receptor isoforms derived by alternative splicing from a single gene in rabbit, cow, mouse, rat, and human (6–10). Within each species, the EP_3 receptor isoforms are identical in the predicted amino acid sequence encoding the seven-TM domains and differ only at the carboxyl termini of the receptor isoforms. Four rabbit EP_3 receptor isoforms, designated 72A, 74A, 77A, and 80A, were cloned from renal cortex (6), although the receptor-evoked signal transduction pathways of these isoforms have not been characterized. It has been demonstrated, however, in the rabbit kidney that PGE_2 elicits a receptor-evoked decrease in $[cAMP]_i$ on binding to endogenously expressed EP_3 receptors (11) .

The EP receptor subtypes are classified by the order of potency of synthetic agonists on smooth muscle preparations. Structure-activity relationships of prostanoid ligands demonstrated that modification of the carboxyl group on C1 reduces PGE_2 agonist potency in a variety of tissues (12). Furthermore, esterification of the C1 of misoprostol-free acid reduced affinity >50-fold at the recombinant G_s-coupled EP_4 receptor (13). It had also been demonstrated that interaction of the C1 carboxylic acid moiety of misoprostol-free acid with prostaglandin receptors was important for potency at the canine EP_3 receptor (14). Although the ligand-binding domain of the EP_3 receptor is largely uncharacterized, analogy with biogenic amine GPCRs suggests that the ligand-binding pocket resides in the putative TM α-helical domains (15). We identified conserved amino acid residues unique to the prostanoid receptor family and hypothesized that these residues are likely to be essential determinants of receptor structure, ligand binding, and/or signal transduction. Residues con-

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[1] Available at http://www.embl-heidelberg.dc

ABBREVIATIONS: PGE_2, prostaglandin E_2; SDS, sodium dodecyl sulfate; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; TM, transmembrane domain; GPCR, G protein-coupled receptor; wt, wild-type, HA, hemagglutinin; HEK, human embryonic kidney; $[cAMP]_i$, intracellular cAMP concentration.

served across the superfamily of GPCRs were presumed to play a role in receptor structure (16–18) .

The ligand-binding and signal transduction properties of the 77A isoform EP_3 receptor were characterized. Affinities of compounds with varying C1 substituents were determined with this cloned EP_3 receptor (77A) expressed in mammalian cells. Also, the effect of the substitution of individual charged amino acid residues in the TM ligand-binding region were examined in terms of binding affinities and receptor-evoked coupling. Taken together, results from these experiments identify potential receptor/ligand interactions as well as residues critical for receptor activation.

Experimental Procedures

Materials. PGE_2, PGE_2 methyl ester, and sulprostone were purchased from Cayman Chemical (Ann Arbor, MI). Misoprostol and misoprostol-free acid were gifts of Dr. Paul Collins (Searle, Chicago, IL). Isoproterenol, ascorbic acid, and indomethacin were purchased from Sigma Chemical (St. Louis, MO). [^{3}H]PGE_2 was purchased from DuPont-New England Nuclear (Boston, MA). Lipofectamine, OPTI-MEM, hygromycin, and G-418 were purchased from Life Technologies (Grand Island, NY). ^{35}S protein labeling mix was purchased from DuPont-New England Nuclear. 12CA5 monoclonal antibody directed against the influenza HA epitope was a gift from Dr. E. Sanders-Bush, Vanderbilt University (Nashville, TN). Protein A/agarose beads were purchased from Pierce Chemical (Rockford, IL). Donkey anti-mouse antibody coupled to Cy-3 was purchased from Jackson/Immunoresearch (West Grove, PA).

Construction of the HA epitope-tagged EP_3 receptor (77A). The HA epitope (YPYDVPDYA) (19) was fused to the amino terminus of the cloned 77A isoform of the rabbit EP_3 receptor using PCR (6). The upstream oligonucleotide primer 5′-CCG GCC GCC ATG TAT CCT TAC GAC GTT CCT GAC TAC GCA CTA GTT CCT CGT CAT ATG AAG GAG ACG CGG GGC-3′ was designed to fuse the HA epitope to the EP_3 receptor. The underlined sequence indicates an *Eag*I restriction site. The first ATG codon marked by overlining represents the initiator codon for the HA epitope. The second ATG codon marked by overlining represents the original 77A initiator methionine codon. This primer was used to amplify 77A template cDNA with *Taq* polymerase with the downstream primer 5′-CAT GTG GCT CGC GTA CCA-3′ at position 477 of the EP_3 coding region, using 95° for 15 sec/60° for 15 sec for 35 cycles and followed by 72° for 10 min. The resulting 549-bp PCR product was subcloned into pCRII, and the nucleotide sequence was confirmed using the dideoxy chain termination method (Sequenase; Amersham, Arlington Heights, IL). The PCR fragment was released by digestion at internal *Eag*I and *Nco*I restriction sites, and the resulting fragment was ligated into the pRc/CMV 77Awt plasmid reconstituting the intact 77A EP_3 receptor/HA epitope fusion. The sequence of the subcloned region in the expression plasmid was verified by dideoxy nucleotide sequencing.

Site-directed mutagenesis of the receptor. Missense mutations were introduced using a sequential PCR protocol. Briefly, two restriction sites were identified flanking the target sequence. In the first reaction, the downstream and upstream oligonucleotides, chosen outside of these restriction sites, were used to amplify a region encompassing the target sequence from 77A template. In the second reaction, a sense oligonucleotide incorporating both the desired amino acid substitution and silent mutations that introduced diagnostic restriction sites was used in conjunction with the downstream oligonucleotide with 77A used as a template, thus incorporating the mutated sequence. Fifty nanograms of each PCR product was mixed and annealed, and the mutated strand was extended using Vent polymerase (New England Biolabs, Beverly, MA) at 95° for 1 min, 55° for 1 min, and 72° for 10 min. The resulting heteroduplex was then reamplified with the flanking upstream and downstream primers at 95° for 45 sec/50° for 45 sec/72° for 45 sec for 35 cycles and at 72° for 5 min. This results in a 50% mixture of the wt and mutated sequence. The PCR fragments were then subcloned into pCRII and screened for novel restriction sites introduced by the mutations. In some mutation experiments, an antisense mutated oligonucleotide was used: upstream (EP_3 nucleotide 914 coding), 5′-ACA TCA GTT GAG CAC TGC-3′; downstream (T7 sequence), 5′-AAT ACG ACT CAC TAT AGG G-3′; R329A, 5′-TC TTA ATA GCT GTT GCC CTG GCT TCA CTG-3′; R329E, 5′-C TTA ATA GCT GTT GAG CTC GCT TCA CTG AAC-3′; D338A, 5′-AAC CAG ATA TTG GCA CCC TGG GTT TAT-3′; and K300A, 5′-CTA CTG ATA ATG ATG CTC GCG ATG ATC TTC AAT-3′. Underlined codon indicates mutated nucleotides. The mutated fragments were then subcloned into 77AHA wt pRc/CMV. The identity of the mutations was confirmed by sequencing both strands of the amplified region according to the dideoxy chain termination method.

Expression of EP_3 cDNAs in cell culture. COS1 cells (8×10^5) were plated and transfected 24 hr later with plasmids encoding the wt or mutant EP_3 receptor (pRc/CMV 77A HA) using 3 μg of plasmid DNA and 36 μg of lipofectamine. Cells were cultured for 72 hr, and the medium was replaced every 24 hr. At 72 hr, cells were lysed, and membranes were prepared as described (6). Protein concentrations were determined with the bicinchoninic acid assay (Pierce).

HEK293tsA201 cells (10^6) were plated and transfected 24 hr later with 0.3 μg of human β_2-adrenergic receptor subcloned into pRc/CMV, 2.7 μg of plasmids encoding the wt or mutant EP_3 receptor (pRc/CMV 77A HA), and 36 μg of lipofectamine. At 24 hr later, cells were plated at 4×10^5 cells/well onto 24-well plates for cAMP studies or cultured for 72 hr in dishes for membrane preparation as described above.

Ligand binding assays. For saturation binding isotherm experiments, 20–100 μg of membrane protein, representing 20 fmol of receptor, was incubated with various concentrations of [^{3}H]PGE_2, and reactions were stopped by filtration through glass-fiber filters as described previously (6). For competition binding assays, 20–40 μg of membrane proteins was incubated with 1 nM [^{3}H]PGE_2 and varying concentrations of unlabeled competitor and processed as described above.

Immunoprecipitation. COS1 cells transfected with various EP_3 cDNAs were grown in cysteine- and methionine-free Dulbecco's modified Eagle's medium/10% fetal bovine serum for 1 hr. Cells were then labeled in medium containing 0.4 mCi/ml ^{35}S-cysteine and ^{35}S-methionine for 4 hr. Cells were lysed in RIPA buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 2 mM phenylmethylsulfonyl fluoride) and incubated for 30 min on ice. The lysate was spun at 100,000 $\times$ *g* for 1 hr, and the supernatants were precleared with protein A/agarose beads in RIPA for 1 hr at 4°. Then, 40 μg/ml anti-HA monoclonal antibody (12CA5) was added to the supernatant and incubated for 12–16 hr at 4°. Receptor protein was precipitated by the incubation with protein A/agarose for 2 hr at 4°. Agarose beads were centrifuged at 12,000 $\times$ *g* for 2 min and washed six times with RIPA buffer, and the receptor protein was eluted with SDS-polyacrylamide gel electrophoresis sample buffer (4% SDS/0.1 M dithiothreitol), with the samples incubated at 37° for 45 min. Immunoprecipitated proteins were resolved on a 10% polyacrylamide gel by SDS-polyacrylamide gel electrophoresis.

Immunofluorescence. Polyclonal transfected COS1 cells were plated onto 24-mm glass coverslips pretreated with 0.01% (w/v) poly-L-lysine. On reaching 80% confluence, coverslips were washed with PBS containing 2 mM $MgCl_2$ and 50 mM $CaCl_2$ and fixed for 30 min in 4% paraformaldehyde in PBS. Cells were then rinsed with PBS permeabilized with 0.2% Triton X-100 in PBS for 15 min and blocked in PBS/2% bovine serum albumin. Cells were incubated with 40 μg/ml 12CA5 for 1 hr at 25°, washed in PBS, and incubated with Cy-3-coupled donkey anti-mouse antibody for 1 hr at 25°. Cells were washed with PBS and mounted on slides for analysis by epifluorescence microscopy at 570 nm.

cAMP measurements. HEK293tsA201 transiently transfected with the human β_2-adrenergic receptor and each 77A HA plasmid were distributed in 24-well plates as described above. At 24 hr later, the medium was aspirated and replaced with 450 μl of Dulbecco's modified Eagle's medium/0.5 mM 3-isobutyl-1-methylxanthine/1 mM ascorbate containing 400 μM indomethacin (to inhibit endogenous prostanoid synthesis) for 1 hr. Medium containing isoproterenol to a final concentration of 1 μM and varying amounts of sulprostone was added to each well and incubated for 15 min. The reactions were stopped by the addition of 500 μl of ice-cold 10% trichloroacetic acid and frozen at −20°. cAMP measurements were performed by radioimmunoassay as described previously (20) .

Data analysis. Saturation binding isotherms, competition binding isotherms, and dose-dependent responses of $[cAMP]_i$ were analyzed using Prism (GraphPAD, San Diego, CA). Statistical analyses were performed using Instat (GraphPAD).

Results

Fusion of the HA tag does not alter EP_3 receptor ligand-binding properties. The EP_3 receptor cDNA was fused to the HA epitope tag as described above to facilitate detection of EP_3 receptor protein expression (Fig. 1). [^{3}H]PGE_2 binding was measured in membranes prepared from COS1 cells transiently transfected with wt or HA-tagged EP_3 receptor cDNAs. The dissociation constants for HA-tagged wt receptor (1.2 ± 0.6 nM) and wt receptor (0.7 ± 0.2 nM) were not statistically significant as determined by an unpaired Student's t test. Furthermore, the affinities of PGE_2 and sulprostone in competition binding isotherms were essentially similar, indicating that fusion of the HA tag to the EP_3 receptor did not appreciably alter its ligand-binding properties (Table 1). Competition isotherms were best fit to a single class of binding sites for each agonist. This absence of high and low affinity agonist-binding sites is consistent with the negligible guanosine-5′-*O*-(3-thio)triphosphate-induced shift in agonist affinity for murine EP3 receptors (9) and observed for the 77A isoform (data not shown).

The methyl ester on C1 of prostaglandin agonists decreases affinity for the EP_3 receptor. Competition binding isotherms performed on membranes prepared from COS1 cells transfected with the HA wt EP_3 receptor demonstrated that misoprostol-free acid had a 150-fold increase in affinity relative to misoprostol, with K_i values of 3.9 ± 1 and 570 ± 130 nM, respectively (Fig. 2). These two compounds differ only in the esterification of the carboxylate moiety on C1, suggesting that the methyl ester carried on the C1 of this PGE analog plays an important role in receptor/ligand interaction. Similar results were obtained for PGE_2 when esterification of C1 of PGE_2 resulted in a K_i value of 780 ± 360 nM for PGE_2 methyl ester, which represents a 370-fold decrease in ligand-binding affinity (Fig. 2 and Table 1).

Mutation of R329, D338, and K300. Fig. 1 shows a schematic of the predicted 77A EP_3 receptor, with highlighted conserved and invariant residues unique to the prostanoid receptors. The 47-amino acid residues identified included three charged residues in the TM regions D95, R329, and D338. Also, although not conserved among all prostanoid receptors, K300 is conserved among EP_3 receptors in each of the five species from which it has been cloned. Residues R329

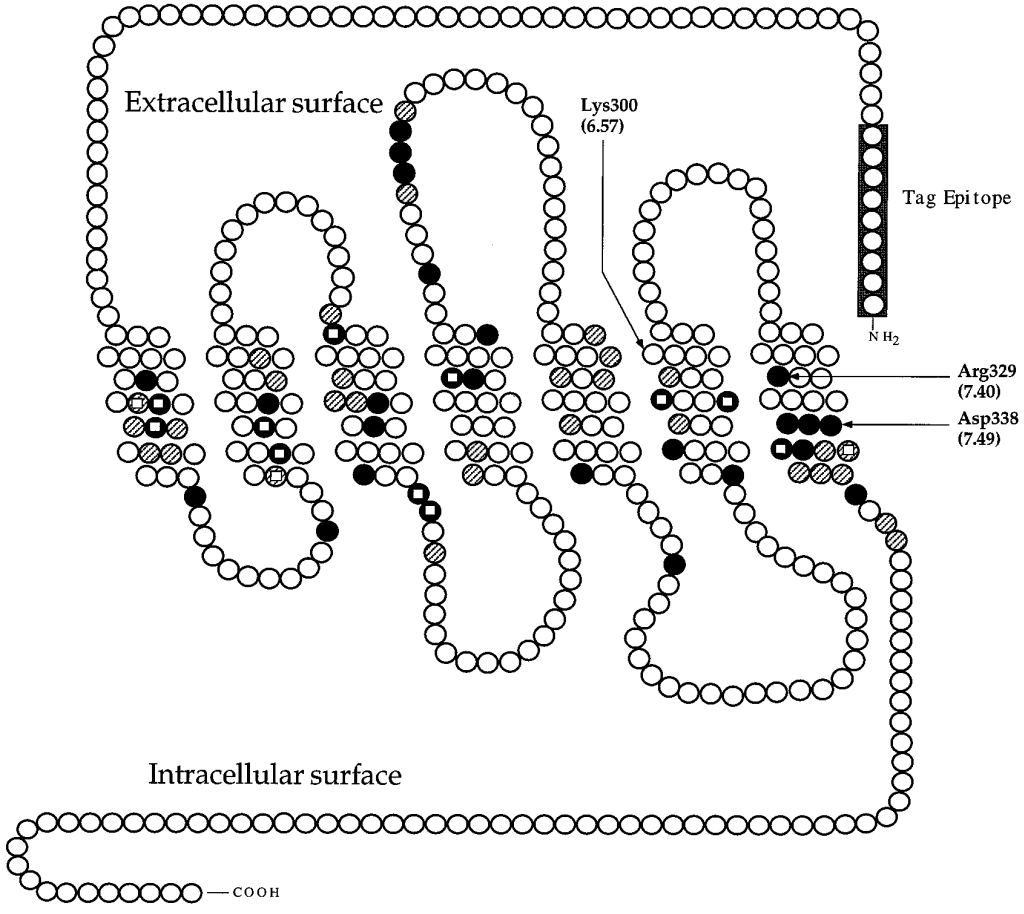

Fig. 1. Identification of conserved residues in the EP_3 receptor. Sequence alignments of the predicted amino acid sequences for the prostanoid receptors were carried out using the TMAP program (39).[1] The sequences aligned were the human and mouse EP_1 receptors; the human EP_2 receptor; the EP_3 receptors for rabbit, rat, mouse, cow, and human; the EP_4 receptors for rabbit, mouse, rat, and human, the thromboxane A_2 receptors for mouse, rat, and human; the FP receptors for mouse, rat, cow, and human; the IP receptors for mouse and human; and the DP receptors for mouse and human. *Shaded circles*, conserved residues; *black circles*, invariant residues. Residues with inset □ are conserved across the entire superfamily of GPCRs (40); those residues without inset □ are unique to the prostanoid receptors. *Parentheses*, target residue GPCR superfamily positions. The amino acid identifier starts with the TM helix number and is followed by the position relative to a conserved amino acid in that TM whose value is arbitrarily set at 50 (17). *Shaded rectangle*, HA epitope fusion to the amino terminus of the receptor.

TABLE 1

Summary of competition binding isotherms of wt and mutant EP_3 receptors expressed in COS1 cells

K_i values were averaged from three independent experiments (± standard error), with the exception of PGE_2 methyl ester binding to KA300 (five experiments).

Receptor	K_i	Agonist
	nM	
HA wt	2.1 ± 0.2	PGE_2
wt (no tag)	0.9 ± 0.2	
D338A	1.5 ± 0.4	
K300A	1.1 ± 0.3	
HA wt	780 ± 360	PGE_2 methyl ester
D338A	730 ± 130	
K300A	3800 ± 1200	
HA wt	3.9 ± 1.0	Misoprostol free acid
D338A	2.1 ± 0.6	
K300A	3.3 ± 0.6	
HA wt	570 ± 130	Misoprostol
D338A	430 ± 130	
K300A	1000 ± 410	
HA wt	0.6 ± 0.2	Sulprostone
wt (no tag)	0.5 ± 0.1	
D338A	0.5 ± 0.1	
K300A	0.5 ± 0.1	

(TM VII), D338 (TM VII), and K300 (TM VI) were mutated, in turn, to alanine to assess the role of these conserved amino acid residues in EP_3 receptor function. In addition, R329 was mutated to a glutamate to test whether the introduction of a negative charge into the putative ligand-binding pocket causes constitutive receptor activation, as has been observed for a similar substitution in bovine rhodopsin (21) .

R329A, R329E, D338A, and K300A refer to the EP_3 receptor proteins fused to the HA epitope tag and bearing these amino acid substitutions (i.e., R329A is an arginine-to-alanine substitution at position 329, and so on). Positively charged TM residues are candidates for interaction with the C1 carboxylic acid moiety of prostanoid ligands. In saturation binding experiments, the R329A and R329E proteins demonstrated a loss of detectable specific $[^3H]PGE_2$ binding when expressed in COS1 cells, suggesting that R329 is important for receptor structure and/or function. In contrast, the K300A mutation located in the extracellular half of the TM VI displayed binding characteristics essentially similar to those of the wt. The K_d value of K300A for $[^3H]PGE_2$ was 1.5 ± 0.1 nM, and the agonist order of affinity was sulprostone > PGE_2 > misoprostol-free acid > misoprostol > PGE_2 methyl ester.

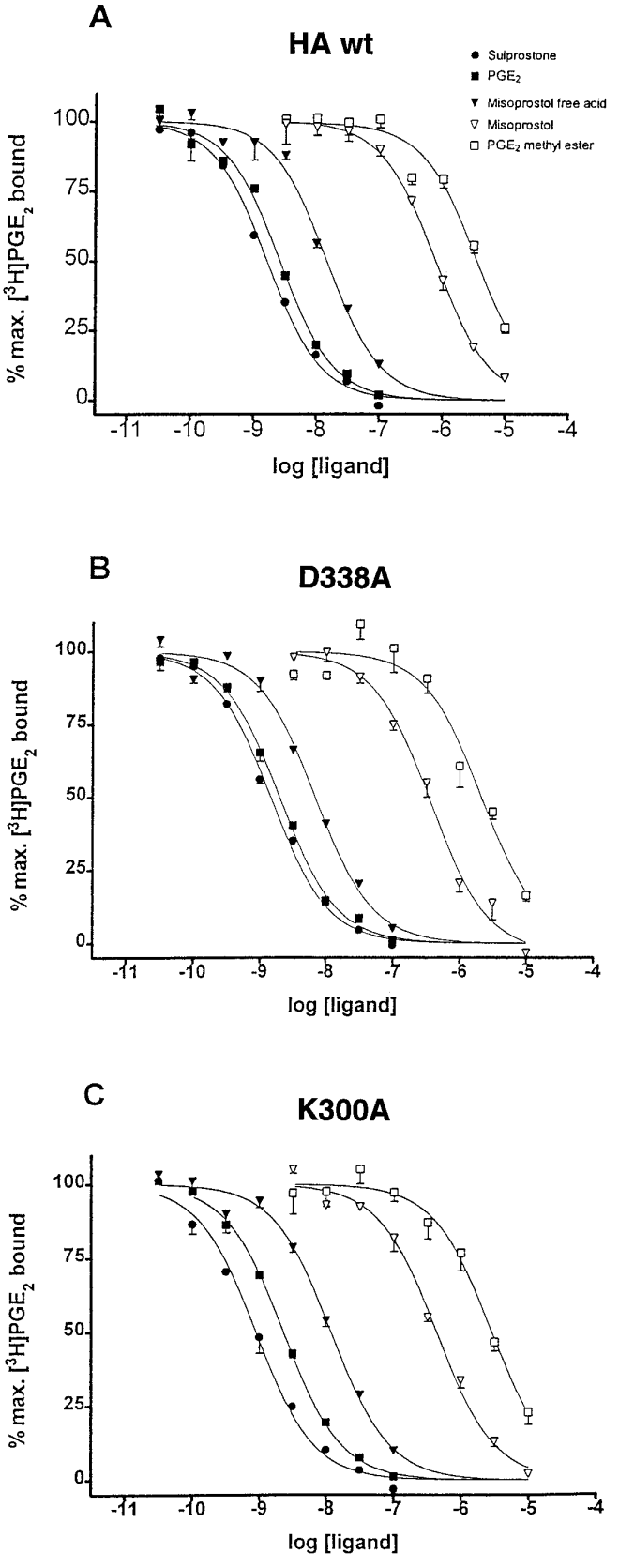

Fig. 2. Competition binding profiles of wt, D338A, and K300A receptors expressed in COS1 cells. Membranes were incubated with 1 nM $[^3H]PGE_2$ and varying concentrations of competitor. Data are from a single experiment performed in triplicate and are representative of three independent experiments.

No significant changes in affinity were observed for K300A compared with the wt receptor (Table 1 and Fig. 2).

A highly conserved sequence in the seventh TM domain was identified in each cloned prostanoid receptor (DPXXY), which corresponds to the conserved NPXXY found in the majority of the GPCR superfamily. The K_d value of D338A for [^{3}H]PGE_2 was 0.9 ± 0.1 nM when expressed in COS1 cells, which is not significantly different from that of the wt receptor. An analysis of variance comparison of the affinities obtained for HA wt, K300A, and D338A demonstrated that there were no statistically significant differences in binding affinities for each of the ligands tested (Fig. 2 and Table 1). The results of the saturation and competition binding isotherms were best described using a one-site-fit model.

Immunoprecipitation of epitope-tagged receptor. The lack of detectable ligand binding by EP_3 receptors bearing R329A or R329E might result from i) a decrease in steady state protein levels, ii) gross perturbations in receptor structure, or iii) elimination of a critical receptor/ligand contact residue. To distinguish among these possibilities, mutant receptor proteins expressed in COS1 cells were immunoprecipitated using a monoclonal antibody directed against the HA epitope tag. Specific protein products of the expected molecular mass for the glycosylated receptor protein (~66 kDa) were detected at similar levels for the wt receptor and each of the mutant receptor proteins (Fig. 3). The precipitation of two species of ~66 kDa is consistent with multiple glycosylated forms of the receptor generated by heterogeneous glycosylation at the four potential *N*-linked glycosylation sites present on the extracellular surface of the protein. No protein was precipitated in the nontagged wt control. These results suggest that each receptor protein was synthesized, glycosylated, and therefore could presumably reach the cell surface (22).

Immunofluorescence. Immunocytochemical analyses of COS1 cells expressing plasmids bearing the wt or mutated cDNAs was performed to further examine the synthesis of wt and mutant receptor proteins. As shown in Fig. 4, these studies suggest that the receptor proteins exhibit similar overall patterns of expression. These experiments were performed in the presence of detergent and thus intracellular as well as plasma membrane-bound receptors were labeled. Taken together with the results of the immunoprecipitation studies, these experiments provide strong evidence that each mutant receptor was expressed at similar levels and had a similar pattern of cellular distribution.

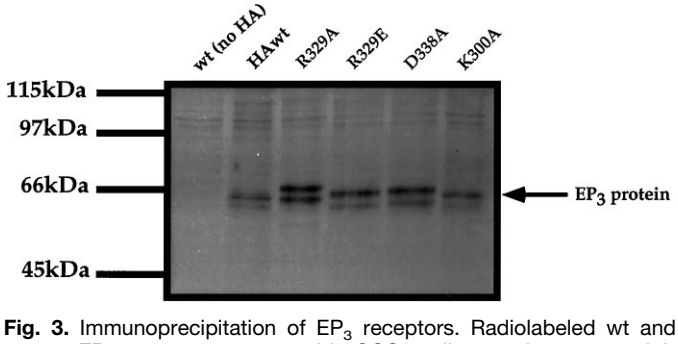

Fig. 3. Immunoprecipitation of EP_3 receptors. Radiolabeled wt and mutant EP_3 receptors expressed in COS1 cells were immunoprecipitated using a monoclonal antibody (12CA5) recognizing the HA epitope fused to the amino terminus of the receptor. *Lane 1*, nontagged wt. All other receptors were fused to the HA tag. *Lane 2*, HA wt. *Lane 3*, R329A. *Lane 4*, R329E. *Lane 5*, D338A. *Lane 6*, K300A. This autoradiogram is representative of three independent experiments.

Signal transduction studies. Plasmids expressing the wt or mutated EP_3 cDNAs were transiently cotransfected with β_2-adrenergic receptor cDNA into HEK293tsA201 cells and stimulated with isoproterenol. K300A and HA wt displayed indistinguishable EC_{50} values for inhibition of $[cAMP]_i$ when stimulated with sulprostone (Fig. 5). In contrast, D338A, R329A, and R329E caused a complete loss of cAMP inhibition at sulprostone concentrations of ≤1 μM, which is 6500-fold higher than the wt EC_{50} value, and the data were best described by a linear fit.

Binding isotherms in HEK293tsA201. To confirm that D338A was expressed in the HEK293tsA201 cells used for signal transduction studies, these cells were also assayed for ligand binding. As shown in Fig. 5, the dissociation constant for [^{3}H]PGE_2 displayed by HA wt, D338A, and K300A receptors expressed in HEK293tsA201 were nearly identical: 1.4 ± 0.4, 1.4 ± 0.4, and 1.1 ± 0.4 nM, respectively. The results of the saturation binding isotherms were best described using a one-site-fit model for each receptor. Furthermore, D338A expressed in HEK293tsA201 bound sulprostone with high affinity (0.6 ± 0.1 nM), indicating that the absence of signal transduction was not due to lack of receptor expression or ligand binding.

Discussion

Studies by Schaaf and Hess demonstrated that the action of PGE_2 on *ex vivo* tissue sample is sensitive to modification of PGE_2 and its analogs at the carboxylic acid moiety at the C1 position (12); however, the individual receptor subtype(s) mediating these effects were undefined. Studies on endogenous EP_3 receptors expressed in canine parietal cells demonstrated that inhibition of endogenous esterase activity decreased the potency of misoprostol, suggesting that prodrug misoprostol is converted to the active compound misoprostol-free acid *in vivo* (14). It has been previously shown that the EP_4 receptor has a 50-fold lower affinity for the subtype-selective ligand misoprostol, which has a methyl ester on C1, compared with its hydrolyzed analog misoprostol-free acid bearing a carboxyl moiety on C1 (13). The EP_3 receptor 77A isoform displays a 150-fold loss in affinity for misoprostol versus misoprostol-free acid, which is attributed to the loss of the charge on C1. Similarly, the EP_3 receptor demonstrated a 370-fold decrease in affinity for PGE_2 methyl ester relative to PGE_2. In contrast, sulprostone has a 3-fold higher affinity than PGE_2. Although the sulfonamide group present on C1 of sulprostone is bulkier than the carboxylic acid moiety, it bears a negative charge (pK_a = 5.25 compared 5.19 for the carboxyl group of PGE_2), supporting the hypothesis that the loss of the negative charge on C1 rather than steric hindrance underlies the decreased affinity observed for compounds with methyl esters.

These data are consistent with interaction of a positively charged amino acid residue within the ligand-binding region of the EP_3 receptor and the C1 carboxylic acid moiety. Bovine rhodopsin also has a positively charged residue in TM VII (K296), which forms a Schiff base with the retinal cofactor. When this lysine was mutated to a glutamic acid, it resulted in loss of retinal binding and constitutive receptor activation (21). It has been proposed that R329 plays a similar role in

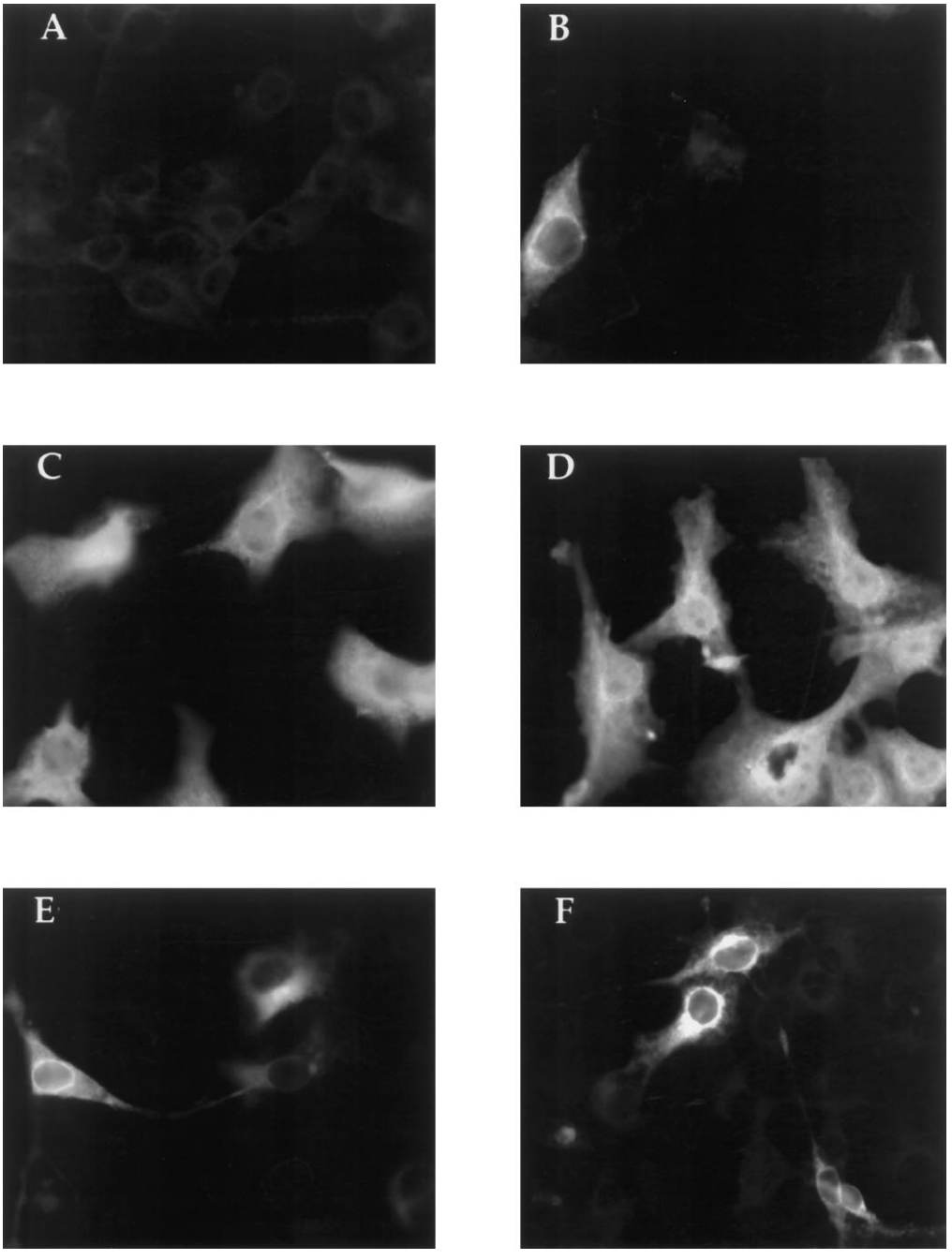

Fig. 4. Immunofluorescence of wt and mutant EP_3 receptors. The wt and mutant EP_3 receptors expressed in COS1 cells were detected using the 12CA5 monoclonal antibody. A, Nontagged wt. B, HA wt. C, R329A. D, R329E. E, D338A. F, K300A. Results are representative of two independent experiments.

the EP receptor family (23). Using the GPCR alignment of van Rhee and Jacobson (17), R329 corresponds to position 7.40 in the EP_3 receptor, whereas K296 of rhodopsin corresponds to position 7.43 and thus is located one helical turn down. Therefore, these residues might not be structurally or functionally homologous. Although the R329A and R329E substitutions did result in a loss of detectable ligand binding, in contrast to rhodopsin, the glutamate substitution at 329 resulted in the loss of detectable EP_3 receptor-evoked signal transduction rather than constitutive activation.

Data presented here suggest that although substitution of K300 does not affect interaction with prostanoid ligands, R329 is a critical determinant of receptor/ligand interaction and signal transduction. Taken together with ligand structure-activity studies, these data are consistent with the interaction of R329 with the C1 carboxylic acid of PGE_2 via either an electrostatic interaction or a charge-stabilized hydrogen bond. An electrostatic receptor/ligand interaction has been suggested for the thromboxane A_2 receptor (24) and the EP_3 receptor (25). Mutation of arginine to glutamine at 7.40 in the bovine EP_3 receptor resulted in small changes in ligand-binding affinity, demonstrating that a positive charge at this position was not required for high affinity ligand binding. This observation, taken together with the results presented here, is more consistent with the interaction of the side chain of R329 with the ligand through a charge-stabilized hydrogen bond.

The sequence NPXX(X)Y in the seventh TM domain is found in >95% of cloned seven-TM GPCRs (16). Site-directed mutagenesis of N322 (residue 7.49) in the β_2-adrenergic re-

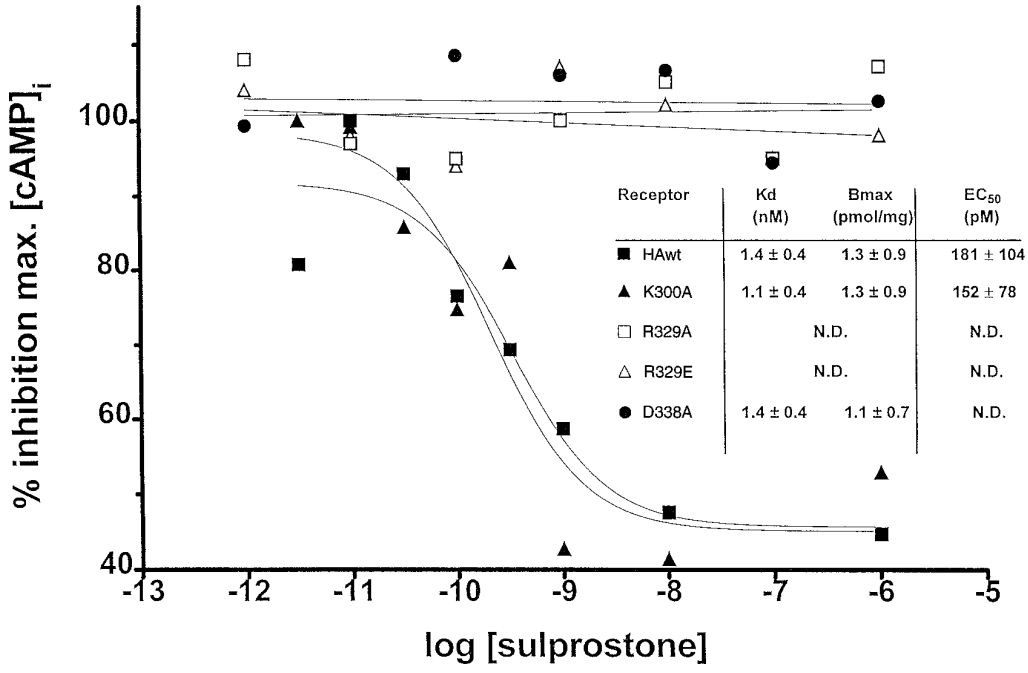

Fig. 5. Sulprostone-mediated inhibition of isoproterenol-stimulated $[cAMP]_i$ production in HEK293tsA201 cells by wt, R329A, R329E, K300A, and D338A. Cells were incubated with various concentrations of sulprostone with 1 μM (−)-isoproterenol for 15 min. The reactions were stopped with the addition of 10% ice-cold trichloroacetic acid, and cell lysates were analyzed for cAMP content by radioimmunoassay. Radioimmunoassay data shown are from a single experiment performed in quadruplicate and are representative of three independent experiments. *Inset*, ligand binding and signal transduction of the wt and mutant receptors expressed in HEK293tsA201 cells. Dissociation constants are the average of three to five independent experiments, each performed in triplicate. *N.D.*, not detected.

ceptor caused a loss in high affinity binding and decrease in efficacy of β_2 agonists in eliciting receptor-mediated signal transduction (26). Similarly, substitution of N298 (7.49) of the AT_1 angiotensin receptor demonstrates that this residue is critical for receptor activation and signal transduction (27). It has been proposed that this conserved asparagine at 7.49 interacts with a conserved aspartic acid at 2.50 in TM II (28). This idea has been supported by results of mutagenesis studies on the gonadotropin-releasing hormone receptor where the residues are reversed, encoding Asn2.50 and Asp7.49 (29). Several groups described a decrease in signal transduction in the gonadotropin-releasing hormone receptor in which aspartate mutates to asparagine at 7.49 (28, 30), suggesting that this amino acid residue may be important for the signal transduction between receptor and G protein. Mutagenesis of both residues (asparagine to aspartate at 2.50 and aspartate to asparagine at 7.49) demonstrated reversion of the signal transduction phenotype, again suggesting an interaction of 2.50 with 7.49 (28). Interestingly, all cloned members of the prostanoid receptor family encode an aspartic acid at both the 7.49 position (D338PWVY in EP_3) and 2.50. In addition to the prostanoid receptors, platelet-activating factor (31) and thrombin receptors (32) also have an aspartic acid at both of these positions. As with the adrenergic and gonadotropin-releasing hormone receptors, we demonstrate here that position 7.49 is a critical residue for signal transduction. It seems unlikely, however, that the two negatively charged groups at 2.50 and 7.49 directly interact with each other.

It has been proposed that interactions of an agonist with its receptor induces specific conformational changes within and between the helices of a receptor and that these events result in a transfer of energy that precedes the activation of G proteins (33, 34). With the assumption that the seventh hydrophobic domain is in an α-helical conformation, D338 is located 180° to R329. If R329 is indeed oriented toward the ligand-binding cleft, D338 faces the lipid environment to some extent. Consistent with this idea, D338A does not appreciably affect high affinity binding of agonists to the EP_3 receptor but rather abolishes the capacity of the receptor to transduce activating signals to effector systems. This phenotype may result from a number of effects: i) Intrahelical flexibility that is necessary for hinge-bending conformational changes partly responsible for receptor activation may be disrupted (35). ii) D338 may represent a key point of interaction between an adjacent helix or helices. Studies on the $5\text{-hydroxytryptamine}_{2a}$ (36), adrenergic (37), muscarinic acetylcholine (38), and gonadotropin-releasing hormone (28) receptors suggest that these interactions represent an essential component of a hydrogen bond network necessary for the proper transfer of energy between helices, a step that is necessary for G protein activation. iii) D338 may interact directly with G_i.

In summary, we have shown by studying the phenotypes of point mutants in ligand binding and signal transduction that R329 (TM VII) and not K300 (TM VI) plays an essential role in EP_3 receptor/ligand interaction and signal transduction and that D338 is essential for receptor-evoked signal transduction but not high affinity binding. Furthermore, these results provide information for the understanding of GPCR activation and the development of a model of the EP_3 receptor.

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