Functional interaction of the carboxylic acid group of agonists and the arginine residue of the seventh transmembrane domain of prostaglandin E receptor EP3 subtype

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Prostaglandin (PG) E_2 binds to PGE receptor EP3 subtype and induces G_i activity. To assess the role of the interaction of the carboxylic acid group of agonists and its putative binding site, Arg-309 in the seventh transmembrane domain of EP3α receptor, in receptor activation, we have mutated the positively charged Arg-309 to the polar but uncharged Gln (EP3α-R309Q) and Asn (EP3α-R309N), and to the non-polar Leu (EP3α-R309L). Wild-type, EP3α-R309Q and EP3α-R309N receptors showed high-affinity binding for PGE_2, but the EP3α-R309L receptor showed very-low-affinity binding. Guanosine 5′-[γ-thio]triphosphate increased the PGE_2 binding to the wild-type receptor, decreased the binding to EP3α-R309Q and EP3α-R309N receptors, but did not affect that to the EP3α-R309L receptor. Furthermore we examined the G_i activities of two types of EP3 agonist, TEI-3356 with a negatively charged carboxylic acid, and TEI-4343, a methyl ester of TEI-3356 with an uncharged but polar group, towards those receptors. Both agonists inhibited the forskolin-stimulated cAMP formation in wild-type, EP3α-R309Q and EP3α-R309N receptors in the same concentration-dependent manner, but the agonists showed a very low inhibition of EP3α-R309L receptor. These findings demonstrate that the hydrogen-bonding interaction of EP3 agonists and residue 309 in the seventh transmembrane domain of the EP3α receptor is sufficient for the functional activation of the EP3α receptor.

INTRODUCTION

Hormonal actions are initiated by agonist binding to receptors. Ligand-binding sites have been extensively studied in various G-protein-coupled rhodopsin-type receptors, suggesting that the ligand-binding sites of G-protein-coupled receptors are contained within the transmembrane domains [1]. However, the chemical nature of the ligand–receptor interactions involved in receptor function is still poorly understood.

Prostaglandin (PG) E_2 produces a broad range of biological actions through its binding to specific receptors on plasma membranes [2]. PGE receptors are divided pharmacologically into four subtypes, EP1, EP2, EP3 and EP4, on the basis of their responses to various agonists and antagonists [3,4]. Among these subtypes, the EP3 receptor has been characterized the best; it has been suggested to be involved in such PGE_2 actions as contraction of the uterus [5], inhibition of gastric acid secretion [6] and sodium and water reabsorption in the kidney tubules [7]. We have cloned the mouse EP3 receptor and demonstrated that this receptor is a G-protein-coupled rhodopsin-type receptor that inhibits adenylate cyclase [8].

Several regions are specifically conserved in the prostanoid receptors; the most highly conserved regions are found in the seventh transmembrane domain [2]. Within the seventh transmembrane domain, the positively charged Arg residue is conserved in all prostanoid receptors [2]. Yamamoto et al. [9] employed molecular modelling to analyse the prostanoid receptor–ligand interaction, and predicted that the Arg residue in the seventh transmembrane domain is the binding site for the carboxylic acid of prostanoids. Consistent with this hypothesis, point mutations at this Arg residue in thromboxane A_2 and EP3 receptors have been similarly shown to result in the loss of ligand-binding activities [10,11]. In these studies the ionic interaction of the carboxylic acid of ligands and the Arg residue of the receptors was suggested to be essential for binding. However, there are various potent EP3 agonists with a variety of esters or methanesulphonamide modifications that can form a hydrogen bond but not an ionic bond [3]. To assess the functional significance of the chemical nature of this interaction, we mutated the positively charged Arg-309 in mouse EP3α receptor to the uncharged but polar Gln and Asn residues and to the non-polar Leu residue. Here we report that the hydrogen-bonding interaction of agonists and EP3α receptor is sufficient for the functional activation of the receptor.

EXPERIMENTAL

Materials

TEI-3356 and TEI-4343 were gifts from Dr. S. Kurozumi (Teijin Ltd.). [5,6,8,11,12,14,15-^{3}H]PGE_2 (179 Ci/mmol) and the ^{125}I-labelled cAMP assay system were obtained from Amersham. PGE_2 was obtained from Cayman Chemical (Ann Arbor, MI, U.S.A.); forskolin was from Sigma, guanosine 5′-[γ-thio]triphosphate (GTP[S]) was from Boehringer Mannheim, and Ro-20-1724 was from Biomol (Plymouth Meeting, PA, U.S.A.).

Construction and stable expression of the mutant receptors

PCR-mediated mutagenesis [12] was employed to replace Arg-309 of mouse EP3α cDNA with Gln, Asn or Leu, and the cDNA species for the respective mutant receptors (EP3α-R309Q, EP3α-R309N and EP3α-R309L) were constructed. cDNA transfection was performed by lipofection, essentially as described previously [13]. Briefly, Chinese hamster ovary (CHO) cells deficient in dihydrofolate reductase activity (CHO-dhfr$^-$) were transfected with the mutated cDNA inserted into the eukaryotic expression

Abbreviations used: CHO, Chinese hamster ovary; GTP[S], guanosine 5′-[γ-thio]triphosphate; PG, prostaglandin.

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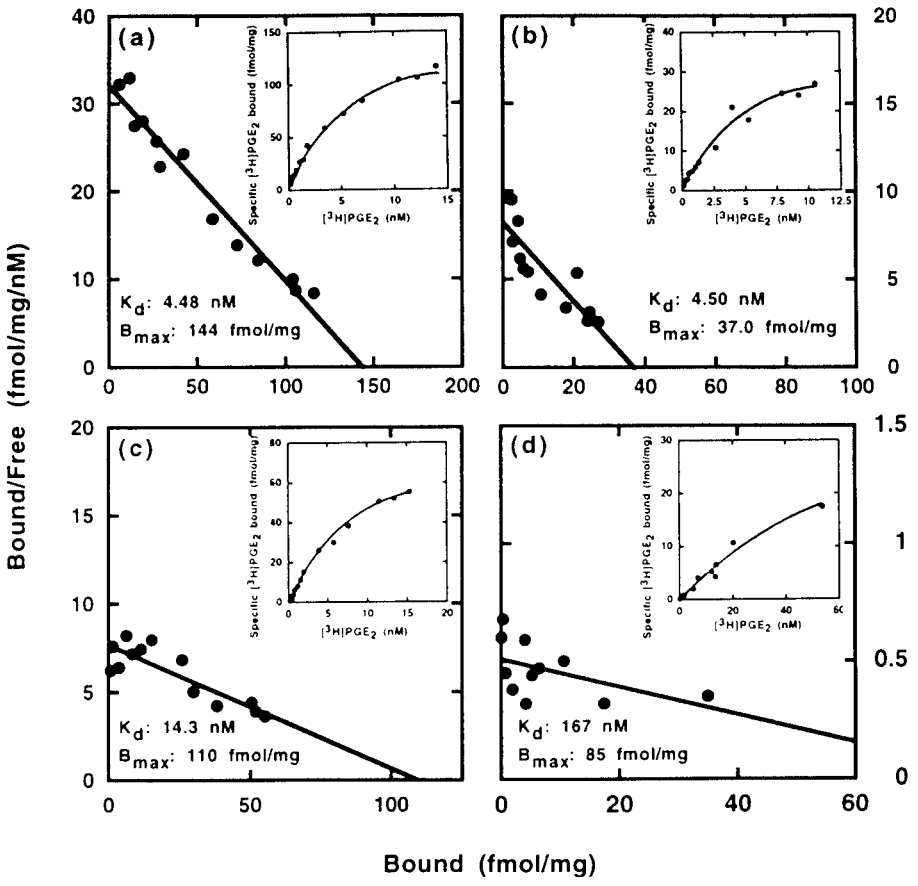

Figure 1 Scatchard analysis of PGE_2 binding to the membrane of CHO cells expressing wild-type or mutant EP3α receptors

The specific binding of [^{3}H]PGE_2 (0.25–80 nM) to the membrane of CHO cells expressing the wild-type (**a**), EP3α-R309Q (**b**), EP3α-R309N (**c**) or EP3α-R309L (**d**) receptor was determined as described in the Experimental section. The Scatchard plot was transformed from the values of specific [^{3}H]PGE_2 binding (inset).

Table 1 Effect of GTP[S] on [^{3}H]PGE_2 binding to wild-type and mutant EP3α receptors

The membrane of CHO cells expressing the wild-type, EP3α-R309Q, EP3α-R309N or EP3α-R309L receptor was incubated with 4 nM [^{3}H]PGE_2 (10 nM [^{3}H]PGE_2 for EP3α-R309L) in the presence (+) or absence (−) of 100 μM GTP[S]. Specific [^{3}H]PGE_2 binding was determined as described in the Experimental section. Results are means ± S.E.M. for three independent experiments.

Receptor	Residue at position 309	Specific [^{3}H]PGE_2 binding (fmol/mg of protein)	
		−GTP[S]	+GTP[S]
Wild-type-EP3α	Arg	50.3 ± 1.5	129 ± 2.8
EP3α-R309Q	Gln	17.5 ± 0.38	7.78 ± 0.57
EP3α-R309N	Asn	21.8 ± 0.66	3.82 ± 0.13
EP3α-R309L	Leu	3.57 ± 0.41	3.81 ± 0.47

vector pdKCR-dhfr containing a mouse dihydrofolate reductase gene as a selection marker [14]. Selection was performed with the α-modification of Eagle's medium, lacking ribonucleosides and deoxyribonucleosides, with 10% (v/v) dialysed fetal bovine serum. Clonal cell lines were obtained by single-cell cloning and were screened by [^{3}H]PGE_2 binding and RNA blotting.

Measurement of cAMP formation

The cAMP contents of CHO cells expressing wild-type and mutant receptors were determined as reported previously [15]. After 10 min of incubation at 37 °C with 10 μM forskolin and 100 μM Ro-20-1724, a phosphodiesterase inhibitor, the reaction was terminated by the addition of 10% (w/v) trichloroacetic acid. The cAMP contents of the cells were determined by radioimmunoassay with an Amersham [^{125}I]cAMP assay system.

PGE_2 binding assay

The harvested CHO cells expressing each receptor were homogenized with a Potter–Elvehjem homogenizer in 20 mM Hepes/NaOH, pH 7.4, containing 10 mM $MgCl_2$, 1 mM EDTA, 20 μM indomethacin and 0.1 mM PMSF. After centrifugation of the suspension at 250 000 ***g*** for 10 min, the pellet was washed, suspended in the same buffer, and used for the [^{3}H]PGE_2 binding assay. The membrane fraction (100 μg) was incubated with various concentrations of [^{3}H]PGE_2 at 30 °C for 1 h, and [^{3}H]PGE_2 bound to the membrane fraction was determined as described previously [16]. Non-specific binding was determined by using a 1000-fold excess of unlabelled PGE_2 in the incubation mixture. Specific binding was calculated by subtracting the non-specific binding from the total binding.

Scatchard plots were transformed from the values of specific [^{3}H]PGE_2 binding, and K_d and B_{max} values were obtained from

linear least-squares curve-fitting by a computer program (radioligand binding analysis program) [17]. K_i values for ligands were calculated with the Cheng–Prusoff equation.

RESULTS

We examined the PGE_2-binding affinities of wild-type, EP3α-R309Q, EP3α-R309N and EP3α-R309L receptors. Figure 1 shows the Scatchard analysis of PGE_2 binding to membrane expressing each receptor. Wild-type, EP3α-R309Q and EP3α-R309N receptors apparently had a single high-affinity binding site. The K_d of the EP3α-R309Q receptor (4.50 nM) was the same as that of the wild-type receptor (4.48 nM), but the K_d of the EP3α-R309N receptor (14.3 nM) was higher than that of the wild-type receptor. In contrast, the EP3α-R309L receptor showed a very low affinity, the K_d being 167 nM. These results demonstrate that the uncharged polar residues Gln and Asn are potent for preserving high-affinity PGE_2 binding, whereas the non-polar residue Leu cannot support high-affinity binding.

Table 2 Effects of GTP[S] on the binding affinities of wild-type and EP3α-R309N receptors for TEI-3356 and TEI-4343

K_d values for PGE_2 were obtained from Scatchard plots of specific [^{3}H]PGE_2 binding to wild-type and EP3α-R309N receptors in the presence (+) and in the absence (−) of 100 μM GTP[S], as described in the Experimental section. K_i values for TEI-3356 and TEI-4343 were obtained from IC_{50} values of 4 nM [^{3}H]PGE_2 binding displacement by both agonists with the use of the Cheng–Prusoff equation. Results are means ± S.E.M. for three independent experiments.

	−GTP[S]		+GTP[S]		
	IC_{50} (nM)	K_i (nM)	IC_{50} (nM)	K_i (nM)	K_i(−GTP[S])/K_i(+GTP[S])
Wild-type					
PGE_2		4.48		1.90	
TEI-3356	100 ± 4.6	52.9 ± 2.5	31.6 ± 1.3	10.2 ± 0.41	5.2
TEI-4343	631 ± 38	334 ± 20	1000 ± 33	322 ± 11	1.0
EP3α-R309N					
PGE_2		14.3		62.5	
TEI-3356	200 ± 8.5	156 ± 6.6	501 ± 33	472 ± 31	0.33
TEI-4343	251 ± 11	196 ± 8.6	708 ± 44	665 ± 42	0.29

The interaction of receptors and G-proteins can be examined by modulation of the receptor-binding affinity by guanine nucleotides [18]. In contrast with most hormone receptors, in which guanine nucleotides decrease the agonist-binding affinity, the PGE_2-binding affinity of EP3 receptor was increased by guanine nucleotides [19]. We then examined the effects of GTP[S] on PGE_2 binding to wild-type and mutant receptors (Table 1). GTP[S] markedly increased PGE_2 binding to the wild-type receptor as expected, but decreased binding to the EP3α-R309Q and EP3α-R309N receptors. In contrast, GTP[S] did not significantly affect PGE_2 binding to the EP3α-R309L receptor. These findings suggest that the difference in the effect of GTP[S] is related to the nature of the interaction of the carboxylic acid group of the agonist and Arg-309 of the EP3 receptor. To investigate further the differential regulation of the agonist-binding affinity by GTP[S], we examined the effects of GTP[S] on the binding affinities for two types of EP3-specific agonist, TEI-3356 and TEI-4343. TEI-3356 contains a negatively charged carboxylic acid, which can interact with the receptor through ionic and hydrogen-bonding interactions. TEI-4343, a methyl ester of TEI-3356, contains an uncharged but polar group, which can interact only through hydrogen bonding [20,21]. Table 2

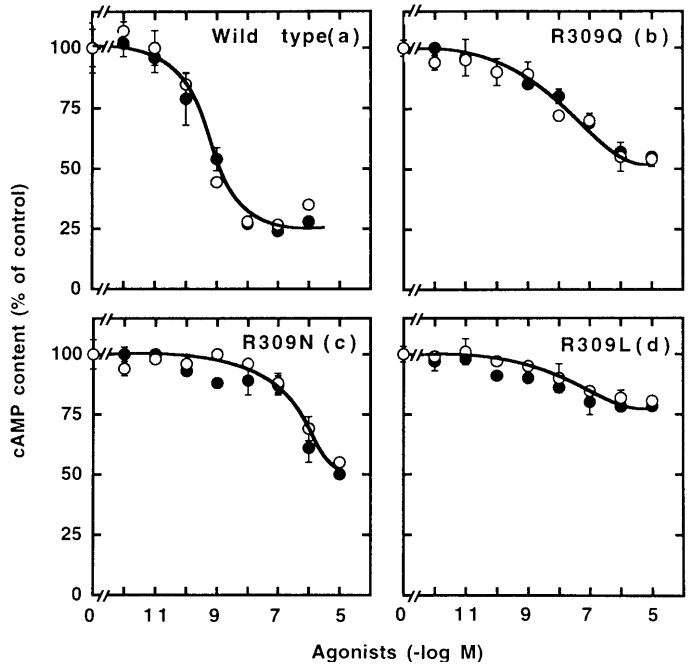

Figure 2 Effects of EP3 agonists on forskolin-induced cAMP formation in CHO cells expressing wild-type or mutant EP3α receptors

CHO cells expressing the wild-type (**a**), EP3α-R309Q (**b**), EP3α-R309N (**c**) or EP3α-R309L (**d**) receptor were incubated for 10 min at 37 °C with the indicated concentrations of TEI-3356 (○) or TEI-4343 (●) in the presence of 10 μM forskolin and 100 μM Ro-20-1724. The cAMP content was measured as described in the Experimental section. Values are expressed as a percentage of the control obtained with the cells in the absence of agonist (wild-type, 7.06 ± 0.64 pmol per well, R309Q; 12.8 ± 0.38 pmol per well; R309N, 13.0 ± 0.21 pmol per well; R309L, 14.8 ± 0.72 pmol per well). The results shown are the means ± S.E.M. for three independent experiments.

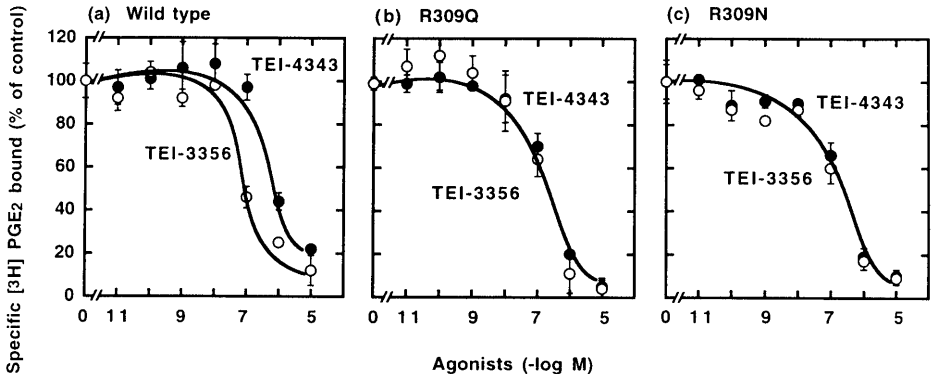

Figure 3 Displacement of [^{3}H]PGE_2 binding by EP3 agonists in the membrane of CHO cells expressing wild-type or mutant EP3α receptors

The membrane of CHO cells expressing the wild-type (**a**), EP3α-R309Q (**b**) or EP3α-R309N (**c**) receptor was incubated with 4 nM [^{3}H]PGE_2 and the indicated concentrations of TEI-3356 (○) or TEI-4343 (●). Specific [^{3}H]PGE_2 binding was determined as described in the Experimental section. Values are expressed as percentages of the control (wild-type, 25.6 ± 2.1 fmol/mg; R309Q, 21.5 ± 0.88 fmol/mg; R309N, 23.5 ± 0.38 fmol/mg). The values are means ± S.E.M. for triplicate experiments.

shows the K_d values of the wild-type and EP3α-R309N receptors for [^{3}H]PGE_2 in the presence and in the absence of GTP[S], and K_i values for TEI-3356 and TEI-4343. GTP[S] markedly increased the binding affinity of the wild-type receptor for TEI-3356 but not for TEI-4343. In contrast, GTP[S] decreased the affinities of the EP3α-R309N receptor for both agonists.

To assess the functional interaction of the carboxylic acid group of the EP3 agonist and its binding site for the EP3 receptor, we examined the G_i activities of two types of EP3-specific agonist, TEI-3356 and TEI-4343. As shown in Figure 2(a), TEI-3356 and TEI-4343 inhibited the forskolin-stimulated formation of cAMP in the wild-type receptor in the same concentration-dependent manner. Both agonists also inhibited the forskolin-stimulated formation of cAMP with the same profile in the EP3α-R309Q and EP3α-R309N receptors (Figures 2b and 2c). The half-maximal concentrations of the agonists for the inhibition were lower in the following order: wild-type < EP3α-R309Q < EP3α-R309N. In addition, their maximal inhibitions (50% inhibition) in the EP3α-R309Q and EP3α-R309N receptors were lower than that (75% inhibition) in wild-type receptor. In contrast, both EP3 agonists showed only 20% inhibition of forskolin-stimulated cAMP formation in the EP3α-R309L receptor (Figure 2d).

We further analysed the binding affinities of the wild-type and mutant receptors for TEI-3356 and TEI-4343 by assessing the displacement of [^{3}H]PGE_2 binding to the receptors. Figure 3(a) shows binding to the wild-type receptor. Both TEI-3356 and TEI-4343 inhibited [^{3}H]PGE_2 binding to the wild-type receptor in a concentration-dependent manner, but the half-maximal concentration of TEI-4343 for inhibition was one order of magnitude higher than that of TEI-3356. In contrast, both agonists showed the same half-maximal inhibition in the EP3α-R309Q and EP3α-R309N receptors (Figures 3b and 3c). We could not obtain displacement curves for both agonists in EP3α-R309L because this receptor exhibited very low [^{3}H]PGE_2 binding at the [^{3}H]PGE_2 concentration used, as suggested by the Scatchard analysis (Figure 1d).

DISCUSSION

In the present study we examined the functional importance of the chemical nature of the interaction between the carboxylic acid group of EP3 agonists and Arg-309 of the seventh transmembrane domain of EP3α receptor. Arg can not only form an ionic pair with the negatively charged carboxylic acid group of ligands, but also donate a hydrogen bond to the carbonyl group of the ligands [22]. Gln and Asn substituted for Arg-309 can form hydrogen bonds with ligands as either a donor or an acceptor of hydrogen bonds but cannot form ionic bonds. However, Leu substituted for Arg-309 is non-polar and can form neither a hydrogen bond nor an ionic bond. In contrast, the carboxylic acid group of PGE_2 has the ability to form either an ionic or a hydrogen bond. The carboxylic acid group of PGE_2 is in equilibrium between the negatively charged form and the uncharged polar one, and is predominantly charged at physiological pH (7.4) [23]. In view of this condition, the binding of PGE_2 to Arg-309 of the wild-type receptor is presumed to be mainly an ionic interaction, whereas the binding to Gln-309 or Asn-309 of the mutant receptor is a hydrogen-bonding interaction. The EP3α receptor with Arg-309 (wild-type), Gln-309 or Asn-309 bound to PGE_2 with high affinity, but the receptor with Leu-309 showed very-low-affinity binding (Figure 1). These results indicate that ionic interaction is not essential for high-affinity binding, and a hydrogen-bonding interaction between PGE_2 and the EP3α receptor is sufficient for the formation of high-affinity binding. Furthermore, because the K_d values of EP3α receptors with Arg-309 and Gln-309 for PGE_2 binding were the same (Figure 1), the high-affinity binding provided by hydrogen-bonding interaction is comparable to that by ionic interaction. In contrast, the K_d value of the EP3α-R309N receptor was three times higher than those of the wild-type and EP3α-R309Q receptors. This decrease in the affinity might be due to the shorter length of the side chain in Asn than in Gln, which weakens the interaction with ligands. Similar results were observed with Asp-113 of the β-adrenergic receptor: replacement of Asp with Glu results in a marked decrease in agonist potency [24].

In most G-protein-coupled receptors, guanine nucleotides decrease agonist-binding affinity to the receptors [18]. However, the EP3 receptor has a unique feature in guanine nucleotide sensitivity: guanine nucleotides increase the agonist-binding affinity [19]. We examined the GTP[S] sensitivities of wild-type and mutant receptors and found that these receptors showed GTP[S]-sensitive PGE_2 binding (Table 1). GTP[S] enhanced PGE_2 binding to the wild-type receptor through ionic interaction but decreased the binding to the EP3α-R309Q and EP3α-R309N receptors through hydrogen-bonding interaction. In contrast,

GTP[S] did not affect the binding to EP3α-R309L, with the non-polar residue. In addition, GTP[S] increased the binding affinity of the wild-type receptor for a negatively charged agonist, TEI-3356, but not for the non-charged analogue TEI-4343, whereas GTP[S] decreased the affinity of EP3α-R309N for both agonists (Table 2). These findings suggest that the ionic interaction of agonist and receptor shows positive sensitivity for GTP[S] and the hydrogen-bonding interaction shows insensitivity or negative sensitivity for GTP[S]. A unique feature of the EP3 receptor in guanine nucleotide sensitivity is related to the ionic interaction of the carboxylic acid of PGE_2 and Arg-309 of the EP3 receptor.

We examined the EP3 receptor-mediated G_i activity by using two types of EP3 agonist, differing only in the structure of the carboxylic acid group. TEI-4343 showed a lower binding affinity than TEI-3356 in the wild-type receptor with Arg-309 (Figure 3a). This lower affinity of TEI-4343 is not due to steric hindrance of the methyl ester moiety, because both agonists showed the same affinities for the mutant receptors with Gln-309 and Asn-309 (Figures 3b and 3c). This finding suggests that TEI-3356 binds to Arg-309 of the receptor through ionic interaction, whereas TEI-4343 binds to the residue through hydrogen bonding. The higher affinity of TEI-3356 for the receptor might in part reflect the different bond strengths of hydrogen and ionic bonds. However, TEI-3356 and TEI-4343 had the same G_i activity in the wild-type receptor (Figure 2a). Thus the ionic bond is not an exclusive interaction for the receptor activation. The hydrogen bonding interaction of agonist and receptor is sufficient for G_i activation. An ionic bond is apparently stronger than a hydrogen bond, but the hydrogen bonding seems strong enough for receptor activation. In addition, both agonists showed the same G_i activities for the two mutant receptors with Gln-309 and Asn-309, which were only capable of forming hydrogen bonds, but their potencies in initiating G_i activity were lower for the mutant receptors than for the wild-type receptor with respect to maximal inhibition and IC_{50} (Figures 2b and 2c). Furthermore, when comparing the two mutant receptors, IC_{50} for the agonist potency in the receptor with Gln-309 was lower than for the receptor with Asn-309. The different agonist potencies in these receptors can be ascribed to different side chain lengths of these residues, the order of side chain length being Arg > Gln > Asn. However, the agonist potencies were only 20% in the mutant receptor with Leu-309, with no ability to form either an ionic or a hydrogen bond. Because Asn and Leu residues have similar side chain lengths, the lower agonist potency in the receptor with Leu-309 is due to the inability to form a hydrogen bond. The EP3α-R309L receptor still has a low level of G_i activity. Multiple binding sites of the receptor for agonists are involved in agonist-induced receptor activation. Although the carboxylic acid group of agonists cannot interact with Leu-309 of the mutant receptor, the interaction through other binding sites might have the ability to induce some extent of G_i activity. These findings indicate that hydrogen-bonding interaction can be important in EP3 receptor-mediated G_i activity. We recently demonstrated that the ionic interaction of the carboxylic acid of PGE_2 and the Arg residue of the seventh transmembrane domain of bovine EP3D receptor, which is coupled to both G_i and G_s, is not essential for G_i coupling but is necessary for G_s coupling [25]. The chemical nature of the interaction of agonist and EP3 receptor might regulate the selectivity of the G-protein coupling.

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We have demonstrated here that the hydrogen-bonding interaction of agonist and EP3 receptor is functional in EP3 receptor-mediated G_i activity and that the chemical natures of the residue at position 309 of the EP3 receptor and the C-1 position of the agonists are key elements in the determination of the high-affinity binding and subsequent activation of the receptor. This study contributes not only to our understanding of the functional interaction of ligand and receptor but will also facilitate the design of therapeutic agents.

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