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Constitutive Activation of the Human Bradykinin B_2 Receptor Induced by Mutations in Transmembrane Helices III and VI

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ABSTRACT

We report that mutation of specific residues in the human B_2 bradykinin (BK) receptor induces its marked constitutive activation, evaluated through inositol phosphate production in COS-7 cells expressing the wild-type or mutant receptors. We provide evidence for a strikingly high constitutive activation of the B_2 receptor induced by alanine substitution of the Asn^{113} residue, located in the third transmembrane domain. These results are reminiscent of our previous finding that mutation of the homologous Asn^{111} residue induces constitutive activation of the AT_1 angiotensin II receptor. BK overstimulation of the constitutively activated mutant N113A receptor was also observed. Phe replacement of the Trp^{256} residue, fairly conserved in transmembrane domain VI of G protein-coupled receptors, also induced a less prominent but significant constitutive activation. Interestingly, the peptidic HOE 140 compound and an original nonpeptidic compound LF 16 0335, which both behaved as inverse agonists of the wild-type receptor expressed in COS-7 cells, became potent and efficient agonists of the two constitutively activated mutant N113A and W256F receptors. These parallel changes observed for two chemically unrelated series can serve as a basis for future studies of structure-function relationships and modeling of activation processes, based on a detailed analysis of the network of helix-helix interactions, which stabilize the inactive receptor conformation and undergo rearrangements on transition to activated states.

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Kinins are biologically active peptides derived from large precursors (kininogens) through the action of serine proteases named kallikreins (Regoli and Barabé, 1980). They produce a number of biological effects, including activation of sensory pain fibers, smooth muscle contraction, endothelium-dependent vasodilatation, and plasma extravasation (Regoli and Barabé, 1980; Proud and Kaplan, 1988; Dray and Perkins, 1993). Kinin-induced responses are mediated by the activation of B_1 or B_2 receptors, which belong to the family of G protein-coupled receptors (GPCRs). Most of the physiological effects appear to be mediated by the activation of B_2 receptors that bind bradykinin (BK) and kallidin (Lys-bradykinin) with high affinities, with the corresponding carboxyl-terminal des-Arg metabolites being agonists of the B_1 receptor.

A number of mutagenesis or biochemical studies on the BK B_2 receptor (Kyle et al., 1994; Nardone and Hogan, 1994; AbdAlla et al., 1996; Jarnagin et al., 1996; Leeb et al., 1997; Fathy et al., 1998, and herein cited references) have been paralleled by sustained efforts to provide molecular models of BK-receptor interactions (Kyle et al., 1994; Jarnagin et al., 1996; Leeb et al., 1997; Fathy et al., 1998). The purpose of the present work was to complete these studies with mechanistic considerations about the receptor conformational changes that underlie the transitions from inactive to active receptor conformations. In this respect, the widely documented phenomena of constitutive activation of GPCR induced by appropriate point mutations (Cohen et al., 1992; Lefkowitz et al., 1993; Noda et al., 1996; Scheer et al., 1996; Groblewski et al., 1997) are useful to predict elements of the network of intramolecular interactions that stabilize the inactive receptor conformation and undergo rearrangements on activation. The finding of B_2 receptor-activating mutations reported in the present work took benefit of previous data from our laboratory relative to the process of activation of the type 1 angiotensin II (AT_1) receptor and the identity in amino acid sequences of the two receptors (about 30%, a high value in the subfamily of peptide hormone GPCR).

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ABBREVIATIONS: AT_1 receptor, type 1 angiotensin II receptor; B_2 receptor, type 2 bradykinin receptor; B_1 receptor, type 1 bradykinin receptor; BK, bradykinin; GTPγS, guanosine-5′-*O*-(3-thio)triphosphate; PLC, phospholipase C; IP, inositol phosphate; WT, wild type; TM, transmembrane domain; GPCR, G protein-coupled receptor; CAM receptor, constitutively activated mutant receptor.

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A preliminary modeling study of the AT_1 receptor pointed out a spatial proximity among the three residues Asp^{74} [transmembrane (TM) II], Asn^{111} (TM III), and Tyr^{292} (TM VII) (Joseph et al., 1995). According to this model, the inactive receptor conformation would involve interaction between Asn^{111} and Tyr^{292}, that is, hormone stimulation would facilitate the disruption of this interaction and allow Tyr^{292} to interact with the conserved Asp^{74}, which was previously shown to be essential for receptor coupling to phospholipase C (Bihoreau et al., 1993). Experimental support to this hypothesis was the finding that $Tyr^{292} \rightarrow$ Phe mutation severely impaired inositol phosphate production induced by angiotensin II (Marie et al., 1994) and that $Asn^{111} \rightarrow$ Ala mutation led to constitutive activation of the receptor (Groblewski et al., 1997). More recently, we refined this model of activation and proposed the involvement of an additional interaction of Asn^{111} with Trp^{253}, located in TM VI (T. Groblewski, J. Marie, and J. C. Bonnafous, unpublished results).

Interestingly, these residues involved in the AT_1 receptor activation are conserved in the B_2 receptor (i.e., Asp^{76}, Asn^{113}, Tyr^{295}, and Trp^{256} for the human receptor; Hess et al., 1992). Our goal was to check whether these residues might play a role in the B_2 receptor activation, as previously found by other authors for the conserved Asp residue in the rat receptor (Quitterer et al., 1996). This comparative study provides a good opportunity to tackle the problem of conservation of molecular events underlying the activation process. The main finding of the present study was the striking constitutive activation of the B_2 receptor induced by mutation of Asn^{113} in TM III. We also found constitutive activation of the B_2 receptor mutated at its Trp^{256} residue in TM VI. Moreover, the constitutively activated mutant (CAM) receptors displayed striking changes in their pharmacological properties: they were overstimulated by peptidic and a nonpeptidic compounds that behaved as inverse agonists of the wild-type (WT) receptor.

Materials and Methods

Reagents and Ligands

BK was purchased from Sigma Chemical (St. Louis, MO). *myo*-[2-^{3}H]inositol and [^{3}H]BK (specific radioactivity ranging around 100 Ci/mmol) were purchased from New England Nuclear (Boston, MA). HOE 140 was kindly supplied by Professor J. Martinez (Centre National de la Recherche Scientifique, Montpellier, France). The nonpeptide derivative LF 16 0335 (1-[[3-[(2,4-dimethylquinolin-8-yl) oxymethyl]2,4-dichloro-phenyl]sulfonyl]2(*S*)-[[4-[4-(aminoiminomethyl)-phenylcarbonyl]]piperazin-1-yl]carbonyl]pyrrolidine) was designed by Fournier Research Laboratories (Daix, France) (Pruneau et al., 1998). COS-7 cells were from the European Cell Type Collection (Salisbury, UK).

Site-Directed Mutagenesis and Receptor Expression

The WT receptor and all mutated receptors were tagged through the addition of a 10-amino-acid epitope from the c-*myc* oncogene at the N termini of receptors truncated at the Asn^3 residue. The cDNA constructs included a Kozak sequence. The various mutations were carried out as described previously (Marie et al., 1994; Groblewski et al., 1997). The WT or mutant receptors were subcloned in the *Eco*RI and *Xba*I sites of the eukaryotic expression vector pCMV polylinker. Receptors were transiently expressed in COS-7 cells by using the electroporation transfection method: 10^7 cells were resuspended in 300 μl of electroporation buffer (50 mM K_2HPO_4, 20 mM KOH, 20 mM CH_3COOK, and 27 mM $MgSO_4$, pH 7.40) and incubated for 10 min at room temperature in an electroporation cuvette (0.4-cm electrode gap; BioRad, Hercules, CA) with 20 μg of pCMV carrier and different amounts of pCMV containing cDNA receptor sequences (2.5–100-ng range). They were submitted to an electric discharge (950 μF, 280 V, 50 ms) and then cultured for 2 days at 37°C in Dulbecco's modified Eagle's medium, 4.5 g/liter glucose, 10% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin before binding or inositol phosphate (IP) accumulation experiments.

Several amounts of pCMV-containing cDNA receptor sequences were systematically used for transfection to compare the properties of receptors expressed at similar levels in the same experiment, a condition required to accurately evaluate constitutive activation phenomena.

Routine evaluation of binding site numbers was carried out using 5 nM [^{3}H]BK in the presence or absence of an excess of unlabeled BK (1 μM).

Binding Assays

Plasma Membrane. Crude membranes from COS-7 cells transiently expressing the WT or mutant B_2 receptors were prepared as described previously (Marie et al., 1994). [^{3}H]BK-binding assays were performed as follows: the membranes were washed twice with binding buffer [25 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid, 140 μg/ml bacitracin, 1 mg/ml bovine serum albumin, 1 mM *o*-phenanthroline, pH set to 6.8 with ammonia] and incubated for 10 min at 30°C in the presence or absence of 100 mM NaCl with or without 100 μM guanosine-5′-*O*-(3-thio)triphosphate (GTPγS) before binding experiments carried out in the same medium. The membranes (15–20 μg/assay) were incubated for 1.5 h at 25°C in binding buffer in the presence of [^{3}H]BK using a 0.05- to 30-nM range. Nonspecific binding was evaluated in the presence of 10^{-5} M unlabeled BK. Bound radioactivity was separated from free ligand by filtration through GF/C filters presoaked in 0.1% polyethyleneimine.

Intact Cells. [^{3}H]BK binding to transfected COS-7 cells grown in 12-well tissue culture clusters (about 2×10^5 cells/well) was carried out at 4°C in Dulbecco's phosphate-buffered saline supplemented with 140 μg/ml bacitracin, 1 mg/ml bovine serum albumin, 1 mM *o*-phenanthroline, and 10^{-5} M captopril, pH 7.0, using a 3-h incubation time, under gentle agitation. Bound radioactivity was evaluated after washing the cells twice with cold binding medium and collecting them in 500 μl of 0.1 N NaOH.

IP Assays

COS-7 cells expressing the WT or mutant receptors were grown in 6-well tissue culture clusters and labeled for 24 h with *myo*-[2-^{3}H]inositol (1.5 ml/well, 1 μCi/ml) in medium 199 deprived of serum. Before stimulation, cells were incubated at 37°C for 1 h in IP buffer [consisting of 116 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 5 mM $NaHCO_3$, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 11 mM glucose, 140 μg/ml bacitracin, and 10^{-5} M captopril, pH 7.4]. After a 15-min preincubation in medium containing 10 mM LiCl, cells were incubated in the presence or absence of ligands for 15 min at 37°C in medium containing 10 mM LiCl. Pooled IPs were extracted and measured as described previously (Paquet et al., 1990). The preincubation step was omitted in the experiments designed to evaluate the properties of HOE 140 and LF 16 0335.

Results and Discussion

To assess the expression of the WT or mutated B_2 receptors and to facilitate their purification, a c-*myc* epitope (10 amino acids) was fused with receptors truncated at the Asn^3 glycosylation site. This modification had no significant influence on the pharmacological properties of the receptors, as as-

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sessed by unchanged K_d values relative to [^{3}H]BK binding in comparative studies carried out on intact COS cells (0.82 ± 0.35 nM and 0.64 ± 0.28 nM for the WT and tagged receptors, respectively; mean of three experiments) and unchanged EC_{50} values for BK-induced IP production (0.48 ± 0.25 nM and 0.51± 0.27 nM for WT and tagged receptors, respectively). The suppression of the potential glycosylation site located near the N terminus, already applied to the AT_1 (J. Marie, unpublished results), might favor tag recognition by a specific antibody. In all described experimental data, "WT receptor" refers to the tagged receptor.

Role of Asn^{76} and Tyr^{295} in B_2 Receptor Activation

Properties of the D76N Mutant B_2 Receptor. The conserved Asp^{76} residue of the human B_2 receptor, which is located in TM II and essential for signal transduction in many GPCR, including the AT_1 receptor (Bihoreau et al., 1993), was mutated into Asn, as in a previous study of the rat B_2 receptor (Quitterer et al., 1996). The binding affinity of [^{3}H]BK to intact transfected COS-7 cells (Table 1) was unchanged in the mutated receptor (K_d = 0.83 ± 0.30 nM) compared with the WT receptor (K_d = 0.54 ± 0.17 nM). The ability of BK to stimulate IP production in cells expressing the mutated D76N receptor was significantly reduced (Figs. 1 and 2). Nevertheless, the residual PLC coupling activity (55%) was surprisingly high if one considers the role generally found for the charged Asp residue. In particular, when carried out in the AT_1 receptor, the Asp → Asn mutation led to either complete (Bihoreau et al., 1993) or almost complete (unpublished results of our laboratory) suppression of phospholipase C (PLC) coupling activity. The dose-response curves relative to BK stimulation (Fig. 2) revealed that Asp → Asn replacement does not modify the EC_{50} value. This partial reduction in PLC coupling is in agreement with recent results relative to the D78N rat B_2 receptor (Quitterer et al., 1996). However, at variance with this latter work, we could not find any significant constitutive activation of the D76N mutant human receptor (Fig. 2). It cannot be excluded that species differences in receptor sequences are responsible for these discrepancies. Mutation of Asp^{76} to Ala further decreased the maximal BK stimulation of IP production (residual activity, 31%) and slightly increased the EC_{50} value (Fig. 2). Nevertheless, the D76A mutant receptor was not constitutively activated, thus confirming that hydrogen bonding involving a residue at position 76 probably is not essential for the stabilization of the inactive receptor conformation.

Role of Na^+ Ions. The following considerations led us to perform a preliminary analysis of the role of Na^+ ions on BK recognition by the WT or mutated human B_2 receptors and receptor coupling to PLC: 1) the well-documented ability of these ions to modulate the GTP effect on receptor–G protein interactions, which usually requires the negative charge of the conserved aspartate in TM II (Kong et al., 1993; Ceresa and Limbird, 1994); 2) the recent work (Quitterer et al., 1996) reporting constitutive activation of the rat B_2 receptor when Na^+ concentration was decreased and proposing that the sodium salt of Asp^{78} corresponds to a very low affinity state for BK, whereas the high-affinity state corresponds to the free aspartate; and 3) our previous finding that the affinity of angiotensin II for the CAM N111A AT_1 receptor was insensitive to Na^+ ion-plus-GTPγS treatment (Groblewski et al., 1997), as a result of profoundly modified coupling properties.

Membranes from COS-7 cells expressing the WT receptor displayed high-affinity binding sites (K_d = 0.47 nM, B_{max} = 0.49 pmol/mg; Table 1) and much lower affinity binding sites (K_d value in the range of 15–20 nM, estimated through decomposition of curvilinear Scatchard plots). Their treatment with 100 mM NaCl lowered the affinity of these high affinity sites by about one order of magnitude (K_d = 2.2 nM, B_{max} = 0.39 pmol/mg). Interestingly, membranes from cells expressing the N113A receptor possessed only high-affinity sites for BK, which were not affected by Na^+ ions (K_d = 0.21 nM, B_{max} = 1.1 pmol/mg in the absence of Na^+; K_d = 0.28 nM, B_{max} = 1.0 pmol/mg in the presence of Na^+) (Table 1). The presence of GTPγS during NaCl treatment did not cause significant additional perturbation of BK recognition by both WT and N113A receptors. The analysis of the D76N mutant receptor (Table 1) revealed that [^{3}H]BK binding to membranes from COS-7 cells expressing the D76N receptor displayed a single

TABLE 1

BK binding properties of the WT and mutant B_2 receptors

The parameters of [^{3}H]BK binding to intact COS-7 cells expressing comparable levels of the WT or mutant receptors, or to membrane preparations from these cells, were carried out as described in *Materials and Methods*. The reported K_d values are the mean ± S.D. of three independent experiments, carried out with triplicate assays. The B_{max} values are those of a typical experiment involving comparison of WT and mutant receptors expressed at similar levels.

B_2 Receptor c-*myc* Tagged	K_d [^{3}H]BK			
	Intact Cells	Membrane		
		No Pretreatment	Na^+ Pretreatment	Na^+ + GTPγS Pretreatment
		nM		
WT	0.54 ± 0.17	0.47 ± 0.17 B_{max} = 0.49 pmol/mg	2.2 ± 0.45 B_{max} = 0.39 pmol/mg	3.7 ± 0.7 B_{max} = 0.38 pmol/mg
N113A	0.67 ± 0.22	0.21 ± 0.11 B_{max} = 1.1 pmol/mg	0.28 ± 0.15 B_{max} = 1.0 pmol/mg	0.27 ± 0.07 B_{max} = 1.1 pmol/mg
D76N	0.83 ± 0.30	0.51 ± 0.15 B_{max} = 1,0 pmol/mg	2.5 ± 0.4 B_{max} = 0,81 pmol/mg	N.D.[a]
W256F	0.52 ± 0.18	N.D.	N.D.	N.D.
W256Q	0.41 ± 0.15	N.D.	N.D.	N.D.
Y295F	0.31 ± 0.13	N.D.	N.D.	N.D.

[a] N.D., not determined.

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class of binding sites in the absence of Na^+ ions (K_d = 0.51 nM, B_{max} = 1.0 pmol/mg). Na^+ ion treatment significantly decreased the affinity of these sites (K_d = 2.5 nM, B_{max} = 0.81 pmol/mg) as observed for the WT receptor.

Taken together, these results indicate that Na^+ ions are somehow involved in receptor coupling, but their effects are complex and do not strictly depend on the integrity of Asp^{76}. Thus our findings on the human receptor are not in agreement with previous results showing that Na^+ ions exert drastic effects on the rat WT receptor by modulating the proportions of high and very low affinity BK binding sites through Asp^{78} sodium salt formation (Quitterer et al., 1996).

Properties of the Y295F Mutant B_2 Receptor. Because the mutation of Tyr^{292} in the AT_1 receptor drastically reduced receptor coupling to PLC (Marie et al., 1994), we checked whether the homologous Tyr^{295}, located in TM VII of the human B_2 receptor, could be involved in its activation process. The Tyr → Phe mutation had no incidence on the receptor affinity for BK (Table 1). Moreover, the receptor coupling to PLC was not significantly affected, as assessed by the unchanged ability of BK to stimulate IP production in COS-7 cells (Fig. 1). This finding constitutes a striking difference between the B_2 and AT_1 receptors (Groblewski et al., unpublished results), indicating that some events associated to their activation processes are not conserved.

Constitutive Activation of the B_2 Receptor Induced by Asn^{113} and Trp^{256} Mutation

Constitutive Activation of the N113A B_2 Receptor. The Asn^{113} residue of the human B_2 receptor, located in its third TM, was replaced by alanine. The WT and N113A mutant receptors were expressed at various levels in COS-7 cells and their coupling to PLC, measured as IP accumulation. The N113A receptor displayed an exceptionally high constitutive activation (up to 40-fold increase in basal activity compared with the WT receptor). Figure 3 shows the basal and BK-stimulated responses of the WT and mutant receptors as a function of their expression levels. The basal activity of the N113A receptor was higher than the maximal BK-stimulated activity of the WT receptor. The mutant receptor could be further activated by ~1.5-fold by saturating doses of BK (Figs. 3 and 4), with a slight increase in the EC_{50} value relative to BK stimulation (0.86 nM versus 0.19 nM for WT, in the typical experiment reported in Fig. 4). The affinities of WT and N113A mutant receptors for [^{3}H]BK, as evaluated in intact cells, were quite similar (K_d = 0.54 ± 0.17 and 0.67 ± 0.22 nM, respectively; Table 1). Strong constitutive activation of the N113A receptor transiently expressed in human embryonic kidney 293 cells was also found (data not shown).

Constitutive Activation of the W256F and W256Q B_2 Receptors. Trp^{256} (TM VI) is conserved in many GPCR, including the AT_1 receptor. Thus Trp^{256} was mutated to phenylalanine or glutamine to preserve the aromatic character of tryptophane or its ability to participate in hydrogen bonding. These mutations did not affect the binding of BK (Table 1). A 3- to 4-fold (up to 10-fold in some experiments) enhancement of basal IP production was reproducibly found for the W256F and W256Q mutants compared with the WT

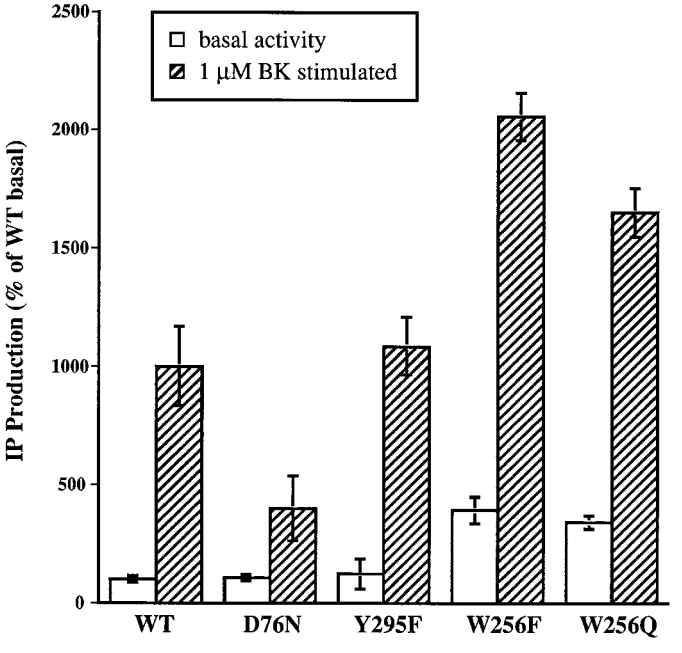

Fig. 1. Basal and BK-stimulated IP production activities of mutant B_2 receptors D76N, Y295F, W256F, and W256Q Basal and 1 μM BK-stimulated IP production activities were measured, as described under Materials and Methods, in COS-7 cells expressing the WT and the various mutant B_2 receptors at comparable expression levels for the comparison, inside the same experiment, of the WT receptor and a given mutant. The WT, W256F, and W256Q receptors were analyzed in the same experiment. The figure represent typical experiments performed in triplicate that are representative of three or more independent experiments relative to each WT/mutant comparison. The expression levels, determined through [^{3}H]BK binding, were all in the range of 1.7 × 10^5 to 2.3 × 10^5 sites/cell.

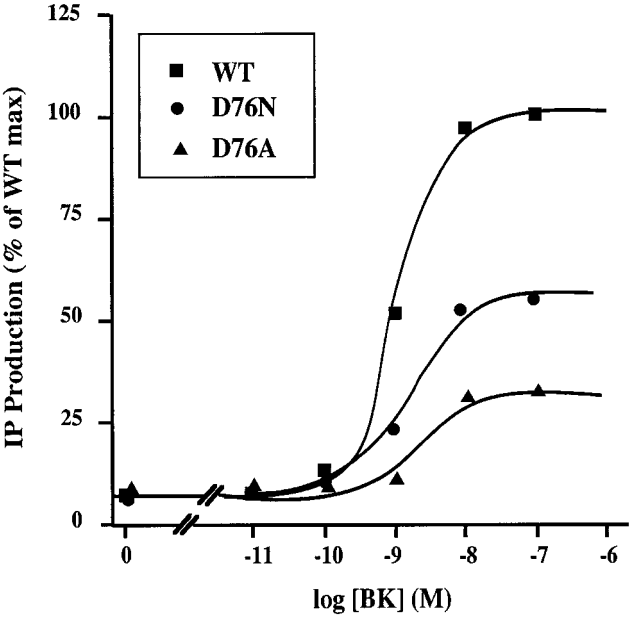

Fig. 2. Dose-dependent BK stimulation of the WT, D76N, and D76A mutant B_2 receptors. The WT and D76N receptors were transiently expressed at comparable levels (1.05 × 10^5 and 1.22 × 10^5 sites/cell, respectively) in COS-7 cells. The stimulation of IP production by varying doses of BK was evaluated as described in *Materials and Methods*. The EC_{50} values were 1.5, 1.6, and 2.5 nM for the WT, D76N, and D76A receptors, respectively, and the maximal BK stimulations of the mutant receptors represented 53% and 25% of that WT receptor. The reported typical experiment is representative of three independent comparative experiments performed in triplicate (mean ± S.D. EC_{50} values = 1.2 ± 0.3, 1.5 ± 0.6, and 2.9 ± 0.9 nM for the WT, D76N, and D76A receptors, respectively; maximal BK stimulations of the asparagine and alanine mutant receptors represented 55 ± 2% and 31 ± 6% of that of the WT receptor)

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receptor expressed at similar expression levels in COS-7 cells, within the same experiment (Fig. 1). The IP response to BK was also increased for the mutated receptors (Fig. 1) without significant changes in the EC_{50} values (0.52 ± 0.18 and 0.41 ± 0.15 nM for the W256F and W256Q receptors, respectively, compared with 0.54 ± 0.17 nM for the WT receptor)

The similar properties of the W256F and W256Q mutant B_2 receptors deserve a comment: the inability of the Trp → Gln mutation to restore a WT constrained conformation could be explained by a spatial localization inadequate for its participation in hydrogen bonding. Refined receptor models, associated to a more systematic mutagenesis scanning at positions 113 and 256, would be required for the interpretation of subtle differences between amino acid side chain orientations in the WT and mutated receptors.

Pharmacological Properties of HOE 140 and the Nonpeptide LF 13 0335 for the WT and CAM Receptors. HOE 140 was originally described as a B_2-specific receptor antagonist (Wirth et al., 1991). Taking into account the inverse agonist properties of this compound for the native B_2 receptor from rat myometrium smooth muscle (Leeb-Lundberg et al., 1994) and its agonist properties on the recombinant rat B_2 receptor expressed in COS cells (Quitterer et al., 1996), we evaluated its ability to modulate IP production in cells transfected with the human WT and the N113A or W256F mutant receptors. In some experiments, we found a basal activity of the WT receptor much higher than that of control untransfected cells. In such situations, we found a strong inverse agonism behavior of HOE140 on the WT receptor, characterized by an almost complete inhibition of the basal activity, with an EC_{50} value of about 2 nM (Fig. 5). On the other hand, HOE 140 was a potent and efficient agonist of the N113A and W256F mutants; its intrinsic activity was 70 to 90% of that of BK, with EC_{50} values in the range of 2 to 3 nM (Fig. 5).

Interestingly, quite similar properties were found for LF16 0335 (see chemical structure in Fig. 6A), a novel potent and selective nonpeptide antagonist of the human B_2 receptor (Pruneau et al., 1998). LF 16 0335 acted as an inverse agonist of the WT receptor (Fig. 6B) and as an agonist of the N113A and W256F CAM receptors (Fig. 6, C and D).

The parallel pharmacological behavior of both CAM receptors provides evidence that their activated states share sufficient structural analogies to be both stabilized (or induced) through their interaction with HOE 140 and the nonpeptide derivative LF16 0335.

The properties of the N113A and W256F human B_2 receptors can be tentatively interpreted in the light of the allosteric ternary complex model proposed by Lefkowitz et al. (1993) and refined by Kenakin (1995). According to this model, the strong constitutive activity of the N113A receptor and, to a lesser extent, of the W256F mutant might indicate that the transition from R to R* was facilitated through the suppression of a structural constraint involving the interactions of Asn^{113} or Trp^{256} with other residues located on neighboring transmembrane helices or, as an interesting possibility, a direct interaction between them. As a consequence of this loss of intramolecular interaction, the energy difference between R* and R would be much lower for the CAM receptor than for the WT receptor, so the ratio $[R^*_{CAM}]/[R_{CAM}]$ is likely to be much greater than $[R^*_{WT}]/[R_{WT}]$. Such an interpretation has been previously provided for a constitutively activated *beta*-2 adrenergic receptor through the construc-

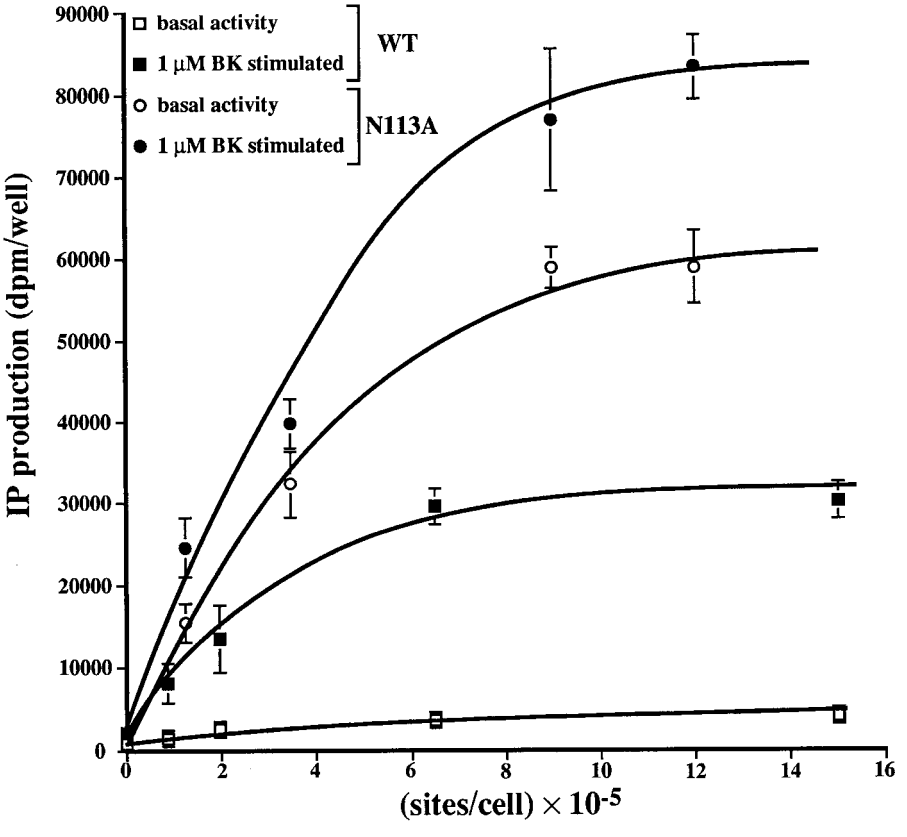

Fig. 3. Constitutive activation of the N113A mutant human B_2 receptor and amplification of its response to BK. Basal and 1 μM BK-stimulated inositol phosphate production were measured in triplicate in COS-7 cells expressing variable levels of WT and N113A mutant B_2 receptors as described in *Materials and Methods*. Mean expression levels in the range 10^5 to 1.5×10^6 sites/cell obtained for variable amounts of specific cDNAs/10^7 electroporated cells were measured through the binding of [^{3}H]BK. The typical experiment described in the figure is representative of 12 independent experiments.

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tion of a simplified thermodynamic diagram (Gether et al., 1997a).

Besides modifications of the constant of isomerization between R and R* and the affinity of R* for the G protein, a point mutation can sometimes induce marked changes in the pharmacological properties of ligands (agonism, antagonism, inverse agonism) (Noda et al., 1996; Groblewski et al., 1997), so parameters accounting for the pattern of a given ligand (Lefkowitz et al., 1993; Kenakin, 1995) are obviously modified on mutation. This might explain the lack of important changes in EC_{50} values relative to BK-activated IP production and the affinities of [^{3}H]BK for the three mutants N113A, W256F, and W256Q compared with the WT receptor. In this respect, a possible loss of a direct interaction between a BK residue and Asn^{113} or Trp^{256} might compensate an affinity increase expected from constitutive activation (Lefkowitz et al., 1993). The construction of a reliable docking of BK into the receptor is required to answer this question.

The experimental data relative to the changes in pharmacological properties of the CAM receptors are consistent with the following assumptions: 1) the inverse agonism properties of HOE 140 and LF 16 0335 for the WT receptor indicate that these compounds preferentially stabilize (or induce) the inactive conformation R_{WT} , and 2) it is difficult to accurately predict structural differences between the activated states R^*_{CAM} and R^*_{WT} and the induction processes involved in the stabilization of R*-ligand complexes. Because HOE 140 and LF 16 0335 are agonists of the CAM receptors, they are expected to preferentially stabilize R^*_{CAM} conformations versus R_{CAM}. These changes in pharmacological properties of WT and CAM receptors will be useful to explore structure-function relationships integrating the preferential recognition and/or induction of receptor conformations by the various ligands.

Conclusion

The present study reports mutations in the human bradykinin B_2 receptor that markedly increase its constitutive activity. It was initiated by previous works from our laboratory on the AT_1 receptor (Marie et al., 1994; Joseph et al., 1995; Groblewski et al., 1997; Groblewski et al., unpublished results) assuming that the extent of amino acid conservation between the two receptors offers the opportunity to study the problem of the conservation of molecular events associated to their activation processes. Interestingly, mutation of Asn^{111} to Ala led to a strong constitutive activation of the AT_1 receptor and to striking changes in the pharmacological properties of some peptidic ligands (Groblewski et al., 1997). We investigated the possible role of its counterpart in the human B_2 receptor and found an exceptionally high constitutive activation on $Asn^{113} \rightarrow$ Ala mutation, characterized by up to 40-fold increases in hormone-independent receptor coupling to PLC and the ability of BK to overstimulate the mutated receptor. The K_d for [^{3}H]BK binding to the N113A receptor was unchanged, which is in agreement with data obtained for the rat receptor (Jarnagin et al., 1996), and the BK-induced overstimulation was characterized by a moderately increased EC_{50} value. One must mention that these results do not preclude the possibility of a direct interaction of BK with Asn^{113}. Indeed, compensating variations of the thermodynamic parameters describing the allosteric ternary complex model can theoretically account for these observations, as already postulated for the interaction of Asn^{111} of the AT_1 receptor with the Tyr^4 residue of the hormone (Joseph et al.,

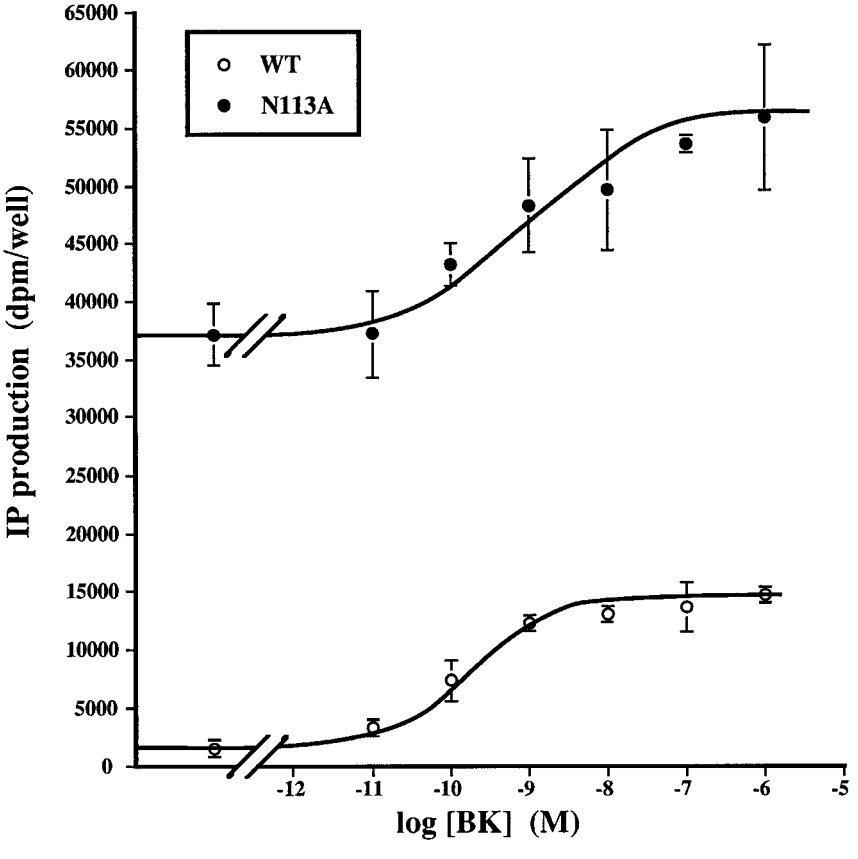

Fig. 4. Dose-dependent BK stimulation of IP production for the WT and N113A B_2 receptors. The stimulation of IP production by various concentrations of BK was analyzed in COS-7 cells expressing the WT or N113A mutant receptors (expression levels = 1.75×10^5 and 2.0×10^5 sites/cell, respectively) as described in *Materials and Methods*. The EC_{50} values in the described typical experiment were 0.19 and 0.86 nM for the WT and N113A receptors, respectively. The curves are representative of three independent experiments (mean EC_{50} values = 0.52 ± 0.35 and 1.2 ± 0.5 nM for the WT and mutant receptor, respectively).

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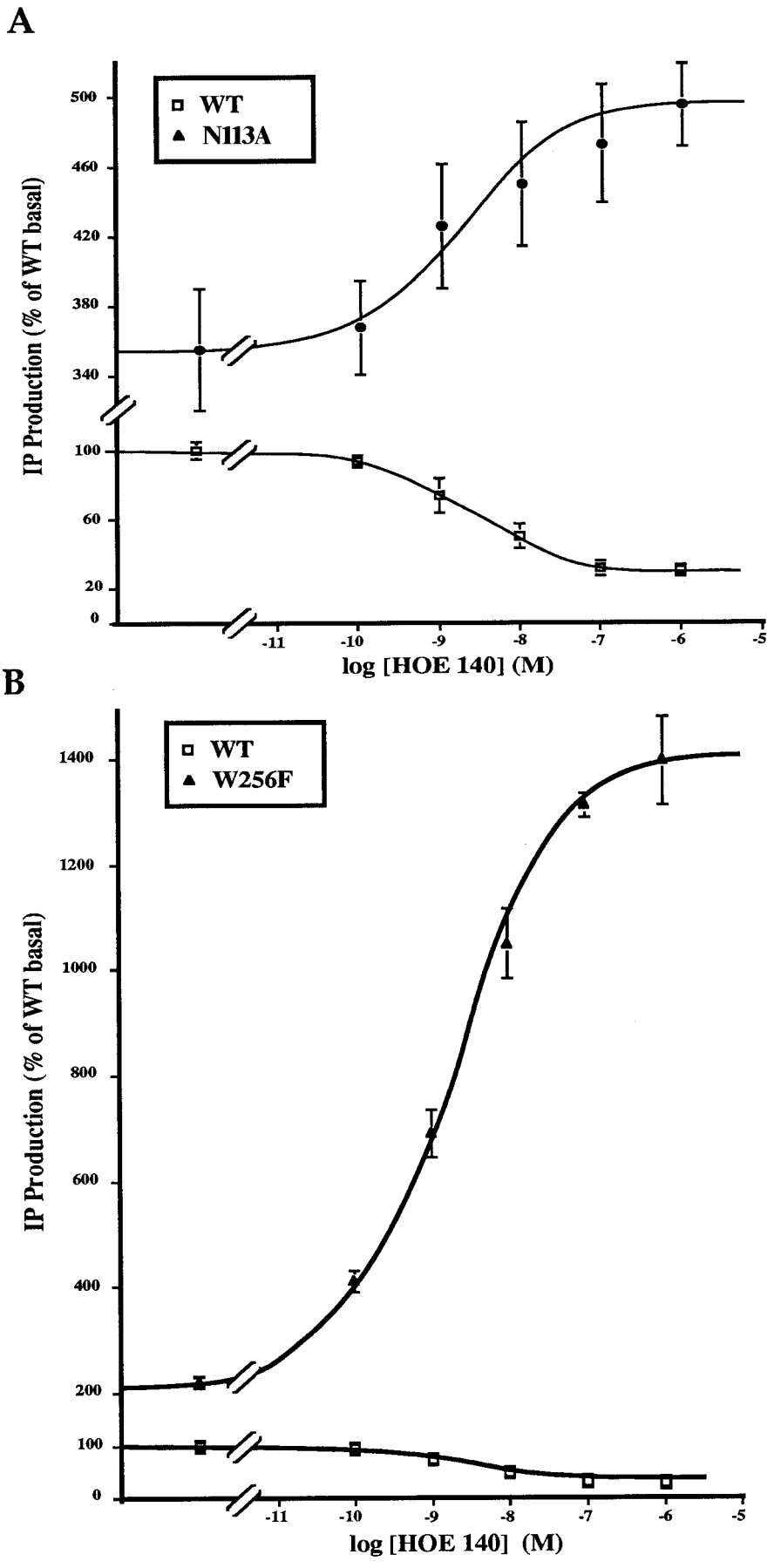

Fig. 5. Modification of the pharmacological properties of HOE 140 on Asn^{113} and Trp^{256} mutation in the human B_2 receptor The effect of varying doses of HOE 140 on IP production was evaluated on the WT, N113A, and W256F mutant B_2 receptors transiently expressed in COS-7 cells as described in *Materials and Methods*. The data represent a typical experiment with triplicate assays and characterized by high basal IP production for the WT receptor (see explanations in *Results* and *Discussion*). A, comparison of the WT and N113A receptor properties: the mean expression levels in the described typical experiment, measured through [^{3}H]BK binding, were 1.2×10^5 and 9.6×10^4 sites/cell for the WT and the N113A mutant receptors, respectively. HOE 140 inverse agonism for the WT receptor and agonism for the N113A receptor were characterized by EC_{50} values of 2.4 and 2.3 nM. respectively (mean ± S.D. value for three separate experiments = 2.3 ± 0.8 and 2.7 ± 0.5 nM for the WT and N113A receptors, respectively). The maximal overstimulation of the mutant receptor by HOE 140 was 88% of the effect of 1 μM BK measured in the same experiment (mean value, 85 ± 11%). The control IP production in nontransfected COS-7 cells represented 18% of the basal IP production by the WT receptor. B, comparison of the WT and W256F receptor properties: the mean expression levels in the described typical experiment measured through [^{3}H]BK binding were 3.0×10^5 and 3.2×10^5 sites/cell for the WT and the W256F mutant receptors, respectively. HOE 140 agonism for the W256F receptor was characterized by an EC_{50} value of 2.3 nM (mean ± S.D. value for three separate experiments = 2.8 ± 0.7 nM). The maximal overstimulation of the mutant receptor by HOE 140 was 75% of that induced by 1 μM BK measured in the same experiment (mean value, 72 ± 13%).

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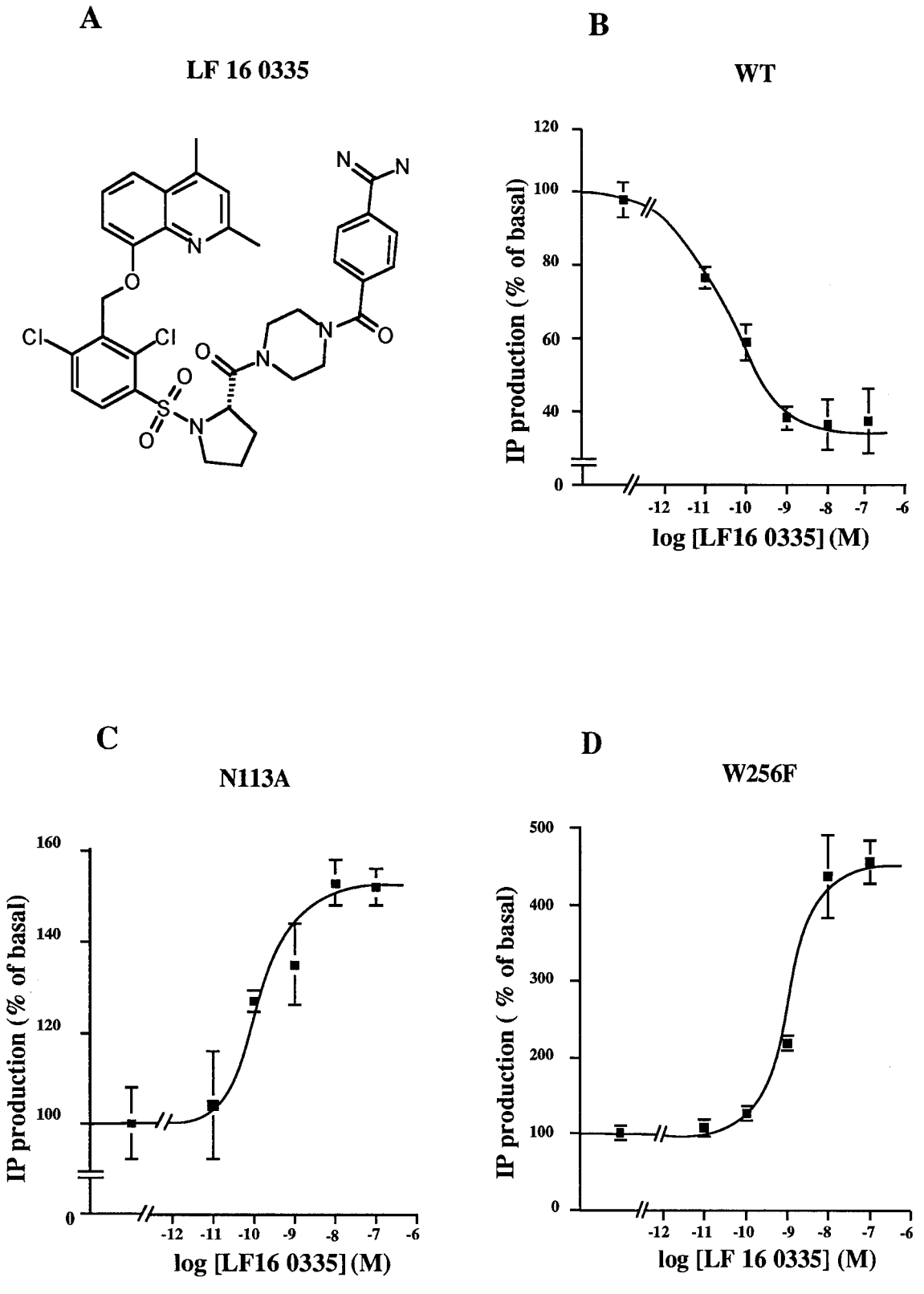

Fig. 6. Pharmacological properties of the nonpeptide LF 16 0335 toward the WT and CAM receptors The effect of varying doses of LF 16 0335 on IP production was evaluated on the WT, N113A, and W256F mutant B_2 receptors transiently expressed in COS-7 cells as described in *Materials and Methods*. The IP production data correspond to a typical experiment and are representative of three separate experiments performed using triplicate assays. A, chemical structure of the nonpeptidic compound LF 16 0335. B, inverse agonist property of LF 160335 for the WT receptor (4.0×10^5 sites/cell) evidenced in a typical experiment on transfected cells displaying high basal IP production. C and D, agonist properties of LF 16 0335 for the CAM receptors N113A (3.8×10^5 sites/cell) and W256F (6.2×10^5 sites/cell): the maximal non-peptide-induced stimulations in the reported typical experiment were 98% and 76% of the BK-induced maximal stimulation, and the EC_{50} values were about 0.1 and 1.8 nM, respectively. The mean ± S.D. values for three separate experiments were EC_{50} = 0.26 ± 0.11 nM and intrinsic activity = 95 ± 6% for the N113A mutant and EC_{50} = 1.6 ± 0.2 nM and intrinsic activity = 74 ± 12% for the W256F mutant.

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1995; Noda et al., 1996). Therefore, an essential conserved property between AT_1 and B_2 receptors is the pivotal role of the Asn residue of TM III. It is noticeable that mutation of the cysteine located at the homologous position in the *alpha*-1B adrenergic receptor also induces constitutive activation (Perez et al., 1996).

Because a recently refined model of the AT_1 receptor (Groblewski et al., unpublished results) suggested a possible interaction between Asn^{111} and Trp^{253}, we also mutated the conserved tryptophane residue in the B_2 receptor. Mutation of Trp^{256} to phenylalanine or glutamine induced 3- to 4-fold increases in basal IP production in transfected COS-7 cells and amplification of the response to BK without any significant perturbation of BK recognition, as previously found for the rat receptor (Jarnagin et al., 1996). It is noteworthy that this tryptophane residue is fairly conserved in the GPCR family and participates in the retinal binding pocket of rhodopsin (Nakayama and Khorana, 1990, Han et al., 1996). The present work constitutes the first report of constitutive activation induced by mutation of this residue.

It is well agreed that constitutive activation of GPCR results from an increase in receptor conformational flexibility caused by the loss of intramolecular bonds (Cohen et al., 1992; Noda et al., 1996; Scheer et al., 1996; Groblewski et al., 1997; and herein cited references). The decreased stability of constitutively activated adrenergic receptors is consistent with this interpretation (Gether et al., 1997; Samama et al., 1997). The analysis of CAM receptor properties is useful to predict some elements of the network of intramolecular interactions that stabilize the inactive receptor conformation and undergo rearrangements on activation. The striking constitutive activation of B_2 receptors mutated at the Asn^{113} or Trp^{256} residues allows formulation of the hypothesis that these residues directly interact in the inactive receptor conformation. Based on similar considerations, a Glu113 (TM III)-Lys296 (TM VII) interaction (Cohen et al., 1992) was taken into account for the building of rhodopsin models. An interaction between the B_2 receptor helices III and VI through an hydrogen bond between Asn^{113} and Trp^{256} would be consistent with the proximity of these helices in rhodopsin (Han et al., 1996). The assumption that this interaction might stabilize an inactive conformation is consistent with recent data indicating that movement of these helices are required for rhodopsin activation (Farrens et al., 1996; Sheikh et al., 1996) as well as data that suggested rotation of helix III (Gether et al., 1997b) and/or helix VI (Gether et al., 1997b; Javitch et al., 1997) participates in *beta*-2 adrenergic receptor activation. Obviously, further investigation, including application of biochemical strategies (i.e., cysteine engineering and locking of privileged conformations through disulfide bonds), is required to unambiguously establish the relative positions of helices III and VI in a B_2 receptor model. It is noteworthy that the transmembrane helices III and VI have been demonstrated to bear residues involved in ligand recognition: role of Phe^{259} and Thr^{263} (TM VI) in BK binding (Nardone and Hogan, 1994; Leeb et al., 1997) and incidence of modifications in helix III on the recognition of B_2-specific ligands (agonist, BK; antagonist, NPC 17731, structurally related to HOE 140) (Fathy et al., 1998). This convergence between experiments dealing with activation and recognition processes obviously requires further refinements to improve the modeling of transmembrane helix bundle movements associated to the transition from inactive (stabilized by antagonists or inverse agonists) to active (stabilized by agonists) B_2 receptor conformations.

The dissection of the molecular events associated with mutation-induced constitutive activation (Cohen et al., 1992; Noda et al., 1996; Scheer et al., 1996; Groblewski et al., 1997) raises the issue of the structural analogy between the activated states for the WT and CAM receptors. It is assumed that agonist activation results from its better recognition of active conformation R* than inactive conformation R. As mentioned by Gether et al. (1997a) for the *beta*-2 adrenergic receptor, the preferential stabilization or induction by BK of the R^*_{CAM} conformation versus R_{CAM} can explain its ability to overstimulate the CAM B_2 receptors, which are characterized by the release of constraints on TM III and TM VI. This process might involve the interaction of BK with helix VI (Leeb et al., 1997), resulting in appropriate positioning for improved coupling. Because modifications in TM III were shown to affect the recognition of B_2-specific ligands (Fathy et al., 1998), the question of direct interaction of BK (or other ligands) with residues in this helix is raised; as mentioned, the involvement of Asn^{113} cannot be ruled out. Similar considerations were taken into account in the quite recent analysis of the activation process of the m_5 muscarinic receptor (Spalding et al., 1998). The comparison of the properties of WT and CAM receptors allows progression toward the understanding of the selection and/or induction processes (Kenakin, 1996) of the various receptor conformations by different ligands. An interesting starting point for these investigations should be provided by the striking differences in the pharmacological properties of the receptors constitutively activated on mutation of Asn^{113} or Trp^{256}. Although the HOE 140 peptidic compound behaves as an inverse agonist of the WT receptor, it becomes a potent and efficient agonist of the N113A and W256F CAM receptors. Similar properties were found for an original nonpeptidic compound, LF 16 0335. Very few examples of mutation-induced changes in GPCR pharmacological properties have been previously reported; they refer to adrenergic (Strader et al., 1989), opioid (Claude et al., 1996), or dopaminergic (Cho et al., 1996) receptors, and it is noteworthy that the pharmacological changes observed in the latter example are obtained through a constitutive activating mutation of a leucine residue neighboring the conserved tryptophane in TM VI. To our knowledge, the present work represents the first example of parallel changes shared by peptidic and nonpeptidic derivatives; moreover, these changes are observed for two receptors mutated at key residues. Future modeling studies will aim at reaching an understanding of the structural basis for the preferential recognition and/or induction by these compounds of the R_{WT} conformation versus R^*_{WT}, whereas they better stabilize or induce R^*_{CAM} than R_{CAM}. The related questions involve the extent of structural analogy between R^*_{WT} and R^*_{CAM} and the precise nature of the molecular events that are shared by the mutation-induced constitutive activations and agonist stimulation of the WT receptor. An essential issue is the precise positioning of transmembrane helices, especially helices III and VI, in these various conformations in the presence or absence of ligands. These structure-function relationship studies will benefit from the availability of many molecules structurally related to the HOE 140 and LF 16 0335 lead compounds.

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Finally, the B_2 receptor, which displays an extreme conformational flexibility on mutation of its Asn^{113} residue and spectacular changes in pharmacological properties on mutation of Asn^{113} and Trp^{256}, constitutes an example of choice for the future study of the dynamic aspect of interconversion between active and inactive conformations. The concomitant analysis of the B_2 and AT_1 receptors offers the opportunity to compare the activation processes of a subclass of peptide hormone receptors with those of rhodopsin or adrenergic receptors.

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