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Inverse Agonism and Neutral Antagonism at α_{1a}- and α_{1b}-Adrenergic Receptor Subtypes

OLIVIER ROSSIER, LILIANE ABUIN, FRANCESCA FANELLI, AMEDEO LEONARDI, and SUSANNA COTECCHIA

Institute of Pharmacology and Toxicology, Université de Lausanne, Lausanne, Switzerland (O.R., L.A., S.C.); Department of Chemistry, Università di Modena e Reggio Emilia, Modena, Italy (F.F.); and Pharmaceutical R&D Division, Recordati S.p.A., Milan, Italy (A.L.)

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ABSTRACT

We have characterized the pharmacological antagonism, i.e., neutral antagonism or inverse agonism, displayed by a number of α-blockers at two α1-adrenergic receptor (AR) subtypes, α_{1a}- and α_{1b}-AR. Constitutively activating mutations were introduced into the α_{1a}-AR at the position homologous to A293 of the α_{1b}-AR where activating mutations were previously described. Twenty-four α-blockers differing in their chemical structures were initially tested for their effect on the agonist-independent inositol phosphate response mediated by the constitutively active A271E and A293E mutants expressed in COS-7 cells. A selected number of drugs also were tested for their effect on the small, but measurable spontaneous activity of the wild-type α_{1a}- and α_{1b}-AR expressed in COS-7 cells. The results of our study demonstrate that a large number of structurally different α-blockers display profound negative efficacy at both the α_{1a}- and α_{1b}-AR subtypes. For other drugs, the negative efficacy varied at the different constitutively active mutants. The most striking difference concerns a group of *N*-arylpiperazines, including 8-[2-[4-(5-chloro-2-methoxyphenyl)-1-piperazinyl]ethyl]-8-azaspiro[4,5] decane-7,9-dione (REC 15/3039), REC 15/2739, and REC 15/3011, which are inverse agonists with profound negative efficacy at the wild-type α_{1b}-AR, but not at the α_{1a}-AR.

Adrenergic receptors (ARs) mediate the functional effects of epinephrine and norepinephrine by coupling to several of the major signaling pathways modulated by guanine nucleotide regulatory proteins (G proteins). The AR family includes nine different gene products: three β (β1, β2, β3), three α_2 (α2A, α2B, α2C), and three α1 (α_{1a}, α_{1b}, α1d) receptor subtypes. Like all G protein-coupled receptors (GPCR), the ARs share seven hydrophobic regions that form a transmembrane α-helical bundle and are connected by alternating intracellular and extracellular hydrophilic loops. Mutational analysis of the ARs has revealed that the α-helical bundle contributes to form the ligand binding site of the receptor, whereas amino acid sequences of the intracellular regions appear to mediate the interaction of the receptor with G proteins as well as with different signaling and regulatory proteins (Wess, 1997).

Both selective and nonselective antagonists for different AR subtypes are widely used in a variety of pathological conditions, including hypertension, heart failure, and prostate hypertrophy as well as in mental diseases such as depression. Several studies have demonstrated that β-blockers can behave either as neutral antagonists or inverse agonists at the wild-type β2-AR or at a constitutively active β2-AR mutant (Samama et al., 1993b; Chidiac et al., 1994). However, inverse agonism at other AR subtypes has been less extensively investigated. It has been previously reported that a small range of α-blockers could inhibit the agonist-independent phospholipase C as well as phospholipase D responses mediated by constitutively active mutants of the α_{1b}-AR (Lee et al., 1997). A recent study demonstrated that some α-blockers can inhibit the spontaneous activity of the α1d-AR subtype (García-Sáinz and Torres-Padilla, 1999).

The main aim of this study was to characterize the pharmacological antagonism, i.e., neutral antagonism or inverse agonism, displayed by a number of α-blockers at two α1-AR subtypes, α_{1a}- and α_{1b}-AR. To achieve this goal, constitutively activating mutations were first introduced into the α_{1a}-AR at the position homologous to A293 of the α_{1b}-AR where activating mutations were previously described (Kjelsberg et al., 1992). Several ligands were then screened for their effect on the agonist-independent activity of both the wild-type α_{1a}- and α_{1b}-ARs and their constitutively active mutants. Our study provides a number of findings that might represent a solid basis to further elucidate the activation process of the α_{1a}- and α_{1b}-AR subtypes, and the mechanism

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ABBREVIATIONS: AR, adrenergic receptor; GPCR, G protein-coupled receptor; DMEM, Dulbecco's modified Eagle's medium; [^{125}I]HEAT, [^{125}I]iodo-2-[β-(4-hydroxyphenyl)-ethyl-aminomethyl]tetralone; IP, inositol phosphate; CAM, constitutively active mutant; R, inactive; R*, active.

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of action of drugs acting at these receptors as well as their structure-activity relationships.

Experimental Procedures

Mutagenesis and Transfections. The cDNA encoding human α_{1a}-AR (Schwinn et al. 1995; cDNA was a kind gift from Dr. J.P. Hieble, SmithKline Beecham, Van Nuys, CA) or hamster α_{1b}-AR (Cotecchia et al., 1992) were mutated by polymerase chain reaction-mediated mutagenesis technique with *Taq* DNA polymerase. The mutated DNA fragments obtained were digested with the appropriate enzymes and cloned into the expression vector pRK-5 containing the wild-type α_{1a}- or α_{1b}-AR cDNA. Recombinant clones were isolated and sequenced. COS-7 cells grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and gentamicin (100 μg/ml) were transfected with the diethylaminoethyl-dextran method. The transfected DNA ranged between 0.5 and 3 $\mu g/10^6$ cells.

Ligand Binding. Membranes derived from cells expressing the α_{1a}-AR subtypes and their mutants were prepared as previously described (Cotecchia et al., 1992). The binding was performed at 25°C in 50 mM Tris-HCl (pH 7.4), 150 mM NCl, and 5 mM EDTA. For saturation binding experiments of [^{125}I]iodo-2-[β-(4-hydroxyphenyl)-ethyl-aminomethyl]tetralone ([I^{125}]HEAT), the radioligand concentration ranged from 12 to 400 pM (150-μl assay volume) and prazosin (10^{-5} M) was used to determine nonspecific binding. For saturation binding experiments of [^{3}H]prazosin, the radioligand concentration ranged from 25 to 4400 pM (300-μl assay volume) and phentolamine (10^{-4} M) was used to determine nonspecific binding. In competition-binding experiments, the final concentrations of [^{125}I]HEAT and [^{3}H]prazosin were 80 and 400 pM, respectively. In some competition-binding experiments, the concentration of [^{125}I]HEAT was 10 pM (500-μl assay volume). Results of ligand binding experiments were analyzed with Prism 2.0 (GraphPAD Software, San Diego, CA).

Inositol Phosphate (IP) Measurement. Transfected COS-7 cells (0.15×10^6) seeded in 12-well plates were labeled for 15 to 18 h with myo-[^{3}H]inositol (New England Nuclear, Boston, MA) at 5 μCi/ml in inositol-free DMEM supplemented with 1% fetal bovine serum. Cells were preincubated for 10 min in PBS containing 20 mM LiCl and then treated for 45 to 100 min with different ligands. Total IPs were extracted and separated as previously described (Cotecchia et al., 1992).

Molecular Modeling of Ligands. The protonated structures of the ligands considered in this study were fully optimized by means of semiempirical molecular orbital calculations (AM1) (Dewar et al., 1985) with the MOPAC 6.0 (QCPE 455) program. QUANTA molecular modeling package (release 96; Molecular Simulation Inc., Waltham, MA) was used for building and analyzing the molecular structures.

Statistical Analysis. Statistical analysis was perfomed as indicated in the figure legends with Prism 2.0 (GraphPAD Software).

Materials. COS-7 cells were obtained from American Type Culture Collection (Rockville, MD). DMEM, gentamicin, fetal bovine serum, and restriction enzymes were purchased from Life Technologies, Inc. (Grand Island, NY). *Taq* polymerase was obtained from Roche Laboratories (RotKruez, Switzerland). [^{125}I]HEAT, [^{3}H]prazosin, and [^{3}H]inositol were obtained from New England Nuclear. (−)-Epinephrine and corynanthine were purchased from Sigma Chemical Co. (St. Louis, MO). 5-Methylurapidil, prazosin, WB 4101, phentolamine, spiperone, *S*-(+)-niguldipine were obtained from Research Biochemicals Inc. (Natick, MA). (+)-Cyclazosin and (−)-cyclazosin were a gift from Dr. D. Giardinà (University of Camerino, Camerino, Italy). Indoramin and AH11110A were a gift from Dr. J.P. Hieble, (SmithKline Beecham), and BE 2254 was a gift from Dr. D. Hoyer (Novartis, Basel, Switzerland). BMY 7378, WAY 100635, SNAP 5089, RS-17053, alfuzosin, terazosin, tamsulosin, REC 15/2739, REC 15/3039 (8-[2-[4-(5-chloro-2-methoxyphenyl)-1-piperazinyl-ethyl]-8-azaspiro[4,5]decane-7,9-dione), REC 15/2869, REC 15/3011, and REC 15/2615 were obtained from Recordati (Milano, Italy).

Results and Discussion

Activating Mutations of α_{1a}- and α_{1b}-AR Subtypes. One strategy to identify inverse agonists is to enhance the basal activity of GPCR by introducing activating mutations and to screen drugs for their ability to inhibit the agonist-independent activity of the constitutively active receptor mutants (CAM). We have previously reported that in the α_{1b}-AR mutations of A293 at the C-terminal end of its 3i loop with any amino acid enhanced the constitutive activity of the receptor, and was highest when alanine was substituted with lysine or glutamic acid (Kjelsberg et al., 1992). To identify inverse agonists at both the α_{1a}- and α_{1b}-AR subtypes, we constructed CAMs of the α_{1a}-AR by mutating A271 (homologous to A293 of the α_{1b}-AR) to lysine or glutamic acid. As shown in Fig. 1, mutations of either A271 or A293 markedly enhanced the basal activty of α_{1a}- and α_{1b}-ARs, respectively, resulting in increased agonist-independent accumulation of IPs. Saturation binding analysis of [^{125}I]HEAT or [^{3}H]prazosin indicated that the expression levels of the wild-type and CAM receptors were good, ranging between 1.7 and 4.3 pmol/mg protein (Table 1). Our findings support the notion previously suggested for other GPCRs (Wess, 1997) that the C-terminal end of the 3i loop plays a crucial role in the conformational switch underlying the transition between the inactive (R) and active states (R*) of the α_{1a}-AR subtype.

Two main differences can be highlighted between α_{1a}- and α_{1b}-ARs. First, the agonist-independent activity of both the wild-type α_{1b}-AR and its CAMs was significantly higher than that of the wild-type α_{1a}-AR or its CAMs. Second, for both the α_{1a}-AR and its CAMs the epinephrine-induced IP accumulation above basal was significantly higher than that of the α_{1b}-AR or its CAMs (Fig. 1). This suggests that the agonist-occupied α_{1a}-AR has greater efficacy in activating phospholipase C than the α_{1b}-AR, whereas its spontaneous or mutation-induced isomerization toward the R* is lower. Our findings are in agreement with those from a previous study (Theroux et al., 1996) describing the coupling efficiencies of

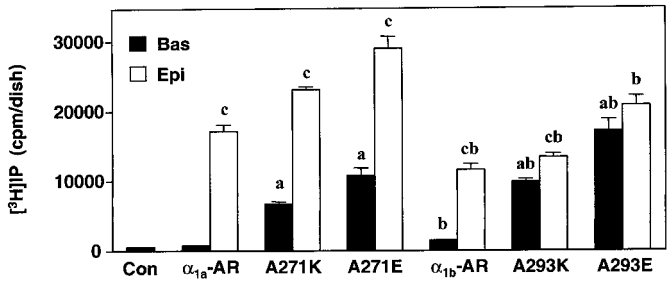

Fig. 1. Constitutively active mutants of the α_{1a}-AR and α_{1b}-AR subtypes. A271 of α_{1a}-AR and A293 of α_{1b}-AR were mutated into lysine and glutamic acid. Receptors expression in COS-7 cells ranged from 1.5 to 2.5 pmol/mg protein. Control (Con) indicates cells not expressing the receptors. IP ([^{3}H]IP) accumulation was measured in cells expressing the wild-type or mutated receptors after incubation in the absence (Bas) or presence of 10^{-4} M epinephrine (Epi) for 45 min. Values represent means ± S.E. of three independent experiments. Statistical significance was analyzed by unpaired Student's *t* test. a, $P < .05$ Bas of the mutants was compared with that of their respective wild-type receptor. b, $P < .05$ Bas or Epi of the α_{1b}-AR, A293K, and A293E were compared with those of α_{1a}-AR, A271K, and A271E, respectively. c, $P < .05$ Epi was compared with Bas of each respective receptor.

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different α1-AR subtypes expressed in HEK 293 or SK-N-MC cells. In that study, the agonist-induced IP response mediated by the α_{1a}-AR was higher, whereas its agonist-independent activity was lower compared with α_{1b}-AR expressed at a similar level.

Inhibition of Receptor-Mediated Basal IP Accumulation. Twenty-four α-blockers differing in their chemical structures were tested for their effect on the basal activity of the constitutively active A271E and A293E mutants expressed in COS-7 cells (Fig. 2). All the ligands used in this study, except REC 15/3039, were previously described for their structure, binding affinity at recombinant as well as native α1-AR subtypes, and some of their pharmacological effects in different tissues (Michel et al., 1995; Giardinà et al., 1996; Leonardi et al., 1997; Testa et al., 1997).

Our results show that the majority of α-blockers displayed inverse agonism as demonstrated by their ability to decrease the basal activity of both CAMs. However, the various α-blockers differed in their negative efficacy and some of these differences depended on the α1-AR subtype.

Drugs with the highest negative efficacy (defined as ≥70% inhibition of the basal activity) at both CAMs included WAY 100635, WB 4101, all the tested quinazolines (prazosin, terazosin, both (+)- and (−)-cyclazosin, REC 15/2615, and alfuzosin), indoramin, corynanthine, spiperone, and AH11110A (Fig. 2). For the other drugs, their negative efficacy differed at the two CAMs.

The most striking difference concerned some *N*-arylpiperazines that displayed modest negative efficacy (e.g., 5-methylurapidil, BMY 7378, and REC 15/2869) or neutral antagonism (e.g., REC 15/3039, REC 15/2739, and REC 15/3011) at the A271E mutant. However, the negative efficacy of these compounds was more pronounced at the A293E, resulting in at least 45% inhibition of the receptor-mediated basal activity. For phentolamine, BE 2254, and tamsulosin negative efficacy was also greater at the A293E than at the A271E. In contrast, for *S*-(+)-niguldipine negative efficacy was greater at the A293E than at the A271E mutant.

The concentration-dependence of the inhibitory effect was determined for those ligands that displayed the most profound negative efficacy at both the A293E and A271E receptors. The EC_{50} values of the compounds in inhibiting the basal activity of the CAMs (Table 2) were in the same order of magnitude as their ligand-binding affinities (Table 3).

To further investigate the mode of action of inverse agonists at the two CAMs we focused on prazosin and 5-methylurapidil whose properties were similar at the two mutants, the first being almost a full inverse agonist and the second having only modest negative efficacy. Treatment of cells with prazosin did not have any effect on aluminum fluoride-induced accumulation of IP (results not shown). The inhibition of basal IP accumulation by prazosin was competitively inhibited by increasing concentrations of 5-methylurapidil. The apparent K_b values of 5-methylurapidil calculated according the Schild equation were in good agreement with its binding affinity for the receptor mutants (Table 3) (K_b = 4.4–5.4 nM and 267–505 nM for the A271E and A293E, respectively). These results confirm that the inhibitory effect of the inverse agonist prazosin is mediated by the receptor and not by other unknown mechanisms on the signaling cascade.

To assess whether the effect of the α-blockers observed on the CAMs reflected their behavior at the wild-type receptors, a selected number of drugs were tested for their effects in COS-7 cells expressing the wild-type α_{1a} and α_{1b}-AR sub-

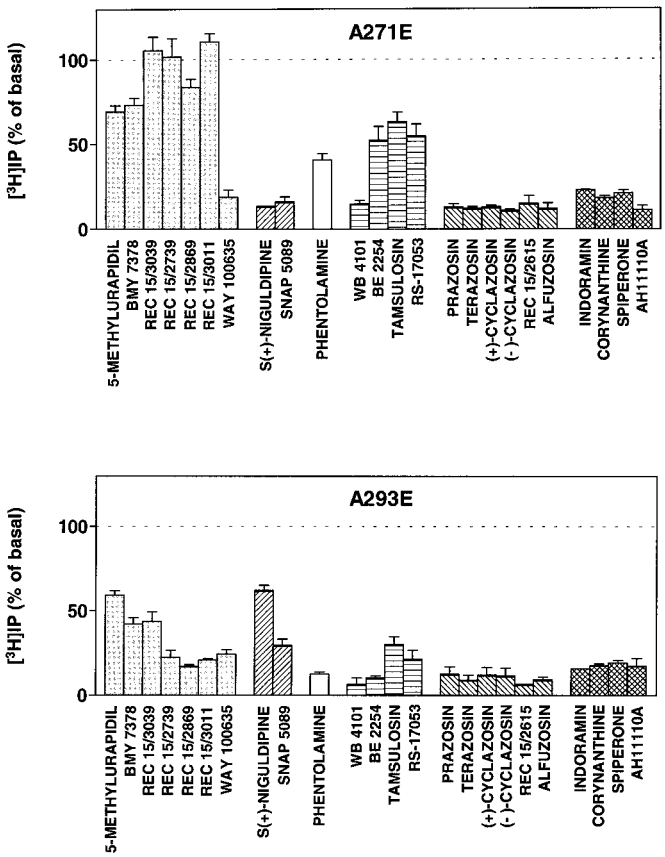

Fig. 2. Ligand-induced inhibition of the basal IP response mediated by constitutively active α1-AR mutants. IP ([^{3}H]IP) accumulation was measuerd in COS-7 cells expressing the A271E and A293E receptor mutants in the absence (basal) or presence of different ligands at a concentration of 10^{-5} M (10^{-4} M for REC 15/3039) for 45 min. The ligands are grouped according to their structural similarities (from left to right, the groups include *N*-arylpiperazines; 1,4-dihydropyridines; imidazolines; benzodioxanes and phenylalkylamines; quinazolines; and various structures). The results are expressed as a percentage of basal, which indicates the basal levels of [^{3}H]IP measured in untreated cells. The basal levels of [^{3}H]IP were as shown in Fig. 1. Values represent means ± S.E. of three to six independent experiments. Statistical analysis with the one-way ANOVA indicated that at both A271E and A293E for all the ligands, the [^{3}H]IP levels were significantly different from basal ($P < .05$) levels with the exception of REC 15/3039, REC 15/2739, REC 15/2869, and REC 15/3011 at the A271E. Statistical analysis with the unpaired Student's *t* test indicated that the percentage of basal of A293E versus A271E was significantly different ($P < .05$) only for REC 15/3039, REC 15/2739, REC 15/2869, REC 15/3011, *S*-(+)-niguldipine, phentolamine, BE 2254, and tamsulosin.

TABLE 1

Expression of the α_{1a}-AR, α_{1b}-AR, and their constitutively active mutants A271E and A293E

[^{125}I]HEAT and [^{3}H]prazosin saturation-binding parameters were determined as described in *Experimental Procedures*. Values are means ± S.E. of three experiments, each performed in triplicate.

Receptor	[^{125}I]HEAT		[^{3}H]Prazosin	
	K_d	B_{max}	K_d	B_{max}
	pM	*pmol/mg*	*pM*	*pmol/mg*
α_{1a}-AR	95 ± 19.8	4.3 ± 0.22	427 ± 63.6	3.4 ± 1.03
A271E	178 ± 44.8	3.4 ± 0.38	567 ± 105.4	2.7 ± 0.98
α_{1b}-AR	132 ± 12.7	3.3 ± 0.85	224 ± 24.0	2.3 ± 0.72
A293E	179 ± 71.15	2.1 ± 0.36	374 ± 60.7	1.7 ± 0.55

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types. Overexpression of both α1-AR subtypes resulted in a small, but measurable increase of agonist-independent accumulation of IP that was greater for the α_{1b}-AR than for the α_{1a}-AR (Fig. 3). As shown in Fig. 3, the effect of the α-blockers in cells expressing the wild-type α1-AR subtypes displayed several similarities to that observed in cells expressing their CAMs. First, the rank order of negative efficacy at the α_{1a}-AR was similar to that observed at the A271E mutant, i.e., prazosin, indoramin, and WB 4101 > BE 2254 > 5-methylurapidil ≥ REC 15/2869 > REC 15/3039, REC 15/2739, and REC 15/3011. Second, all drugs displayed pronounced negative efficacy at the α_{1b}-AR as previously observed for the A293E mutant. Thus, the only ligands that did not display any inverse agonism at the wild-type α_{1a}-AR were REC 15/3039, REC 15/2739, and REC 15/3011, the first two being neutral, whereas REC 15/3011 displayed some nonsignificant degree of partial agonism.

For all the *N*-arylpiperazines tested (5-methylurapidil and the REC compounds) negative efficacy seemed more pronounced at the wild-type α_{1b}-AR (Fig. 3) than at its constitutively active mutant A293E (Fig. 2). For few *N*-arylpiperazines, including 5-methylurapidil and REC 15/2869 as well as for BE 2254 negative efficacy also seemed more pronounced at the wild-type α_{1a}-AR (Fig. 3) than at its CAM A271E (Fig. 2). This observation might find some explanation in the framework of the allosteric ternary complex model describing GPCR activation (Samama et al., 1993a). Activating mutations are suggested to increase the isomerization constant (J) of the receptor allowing its transition from the R to R* states, thus resulting in its high spontaneous activity. Therefore, the negative effect of inverse agonists on receptor isomerization might be larger for the wild-type receptor, which is probably characterized by a very small J parameter, compared with its CAM for which the value of J should be much greater. This might explain why for compounds with some degree of negative efficacy, i.e., 5-methylurapidil, some REC compounds, and BE 2254, inverse agonism was more pronounced at the wild-type α1-AR subtypes than at their CAMs. In contrast, the fact that the behavior of REC 15/3039 and REC 15/2739 was very similar at the wild-type α_{1a}-AR compared with its A271E mutant strongly suggests that their efficacy is truly close to zero at the α_{1a}-AR subtype.

TABLE 2

EC_{50} values of inverse agonists on the inhibition of basal IPs

Concentration-dependent inhibition of basal IP accumulation was measured in COS-7 cells expressing the constitutively active A271E and A293E mutants. Values are means of two to six independent determinations done in triplicate that did not differ by >40%.

Ligand	EC_{50}	
	A271E	A293E
	nM	
Indoramin	5.48	187
Prazosin	1.20	1.09
Terazosin	12.0	8.90
WB 4101	7.12	38.3
Tamsulosin	N.D.	2.03
Phentolamine	4.68	47.5
Spiperone	57.7	79.9

N.D., not determined.

TABLE 3

Ligand binding properties in cells expressing the α_{1a}-AR, α_{1b}-AR and their constitutively active mutants A271E and A293E

K_i values were determined in competition binding experiments with 80 pM [^{125}I]HEAT in membranes from COS-7 cells expressing the various receptors. The best fit of the competition curves was monophasic and the Hill coefficient ranged from 0.7 to 1. Values are means of two to four independent determinations that did not differ by >40%.

Ligand	K_i			
	α_{1a}-AR	A271E	α_{1b}-AR	A293E
		nM		
5-Methylurapidil	5.33	8.65	367	521
REC 15/3039	1131	606	1650	1019
REC 15/2739	2.24	2.15	121	218
REC 15/2869	24.1	12.5	342	528
REC 15/3011	15.2	6.35	442	607
Indoramin	19.4	31.7	189	215
RS 17053	6.93	4.79	51.0	50.0
Prazosin	0.92	1.02	0.69	0.57
Terazosin	33.8	35.8	19.1	15.5
WB 4101	0.99	1.11	16.2	21.1
Tamsulosin	0.19	0.21	8.53	7.94
Phentolamine	9.71	13.3	183	236
Spiperone	57.6	76.2	16.1	23.4

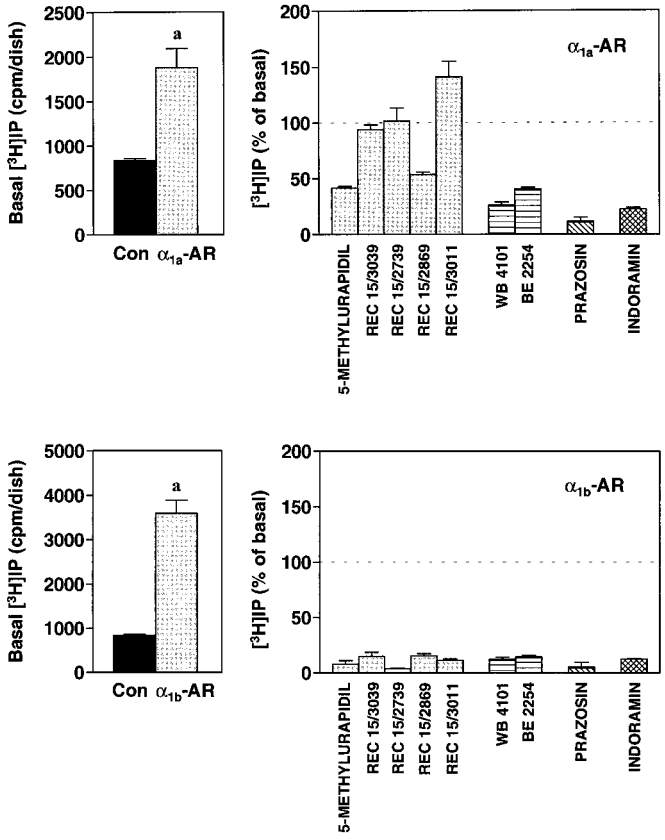

Fig. 3. Ligand-induced inhibition of the basal IP response mediated by the wild-type α_{1a}-AR and α_{1b}-AR. The left-hand graphs show the basal [^{3}H]IP accumulation in COS-7 cells expressing the wild-type α_{1a}-AR and α_{1b}-AR after a 100-min incubation in the absence of ligands. Receptor expression ranged from 2 to 3 pmol/mg protein. Control (Con) indicates cells not expressing the receptors. Statistical significance was analyzed by unpaired Student's *t* test. a, $P < .05$ Bas of cells expressing the receptor versus control cells. The right-hand graphs show [^{3}H]IP measured in cells expressing the α_{1a}-AR and α_{1b}-AR treated with different ligands at a concentration of 10^{-5} M for 100 min. The ligands are grouped as in Fig. 2. The results are expressed as a percentage of basal, which indicates the basal levels of [^{3}H]IP measured in untreated cells. Values represent means ± S.E. of three independent experiments. Statistical analysis with one-way ANOVA indicated that at both α_{1a}-AR and α_{1b}-AR for all the ligands, the [^{3}H]IP levels were significantly different from the basal ($P < .05$) with the exception of REC 15/3039, REC 15/2739, and REC 15/3011 at the α_{1a}-AR.

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Collectively, these findings identify a group of *N*-arylpiperazines as α-blockers that display the most striking difference at the two α1-AR subtypes being inverse agonists with profound negative efficacy at the wild-type α_{1b}-AR, but not at the α_{1a}-AR. In particular, REC 15/3039, REC 15/2739, and REC 15/3011 are the first α-blockers identified so far that do not display inverse agonism at one of the α1-AR subtypes, namely, the α_{1a}.

Structure-Activity Relationships of Ligands. A typical α-blocker contains aromatic moieties on each side of a protonated nitrogen atom, one of these two moieties being closer to the protonated nitrogen than the other. The protonated nitrogen atom of the ligands is thought to interact with the conserved aspartate on helix 3 of the receptor, i.e., D106 in the α_{1a}-AR and D125 in the α_{1b}-AR, according to a precise geometry dictated by a directional charge-reinforced hydrogen bonding interaction (Cavalli et al., 1996; De Benedetti et al., 1997). The geometry of the electrostatic interaction between the protonated nitrogen of the ligand and the aspartate of the receptor probably dictates the orientation of the whole ligand molecule within the receptor binding site, thereby generating a peculiar local perturbation that is transferred to the receptor domains involved in G protein coupling. Thus, a conformational link existing between the receptor binding site for various ligands and its interaction site with the G protein might dictate the different functional effects of ligands on distinct receptors.

Our findings indicate that α_{1a}-AR and α_{1b}-AR differ in their "susceptibility" to inverse agonism. Whereas various ligands displayed different effects on the α_{1a}-AR, most of the ligands tested exerted a profound inhibitory effect on the agonist-independent activity of the α_{1b}-AR, independently from their structural features. This divergence might be related to the different configuration of the binding site of the two α1-AR subtypes, as reported in our previous molecular modeling studies (De Benedetti et al., 1997). One might speculate that compared with α_{1a}-AR, α_{1b}-AR has a larger number of "inhibitory sites", i.e., sites mediating inverse agonist-induced receptor inactivation, or that structurally different ligands may invariantly reach the "inhibitory sites" because of the less flexible configuration of the receptor binding site.

Molecular modeling of the ligands provided some interesting insight into the structure-activity analysis of the α-blockers (Fig. 5). For simplicity, among all the α-blockers used in this study (Fig. 4) only the models of those representative of some best defined structural groups are shown in Fig. 4. The inhibitory effect observed for different ligands on the constitutive activity of the α_{1a}-AR and, to a lesser extent, on that of the α_{1b}-AR seems to be related to the structure of a defined portion of the ligands. Our results suggest that the geometry of the protonated nitrogen atom as well as that of the molecular moiety closest to this nitrogen might be responsible for the functional effect of the ligands tested (Fig. 5).

On the basis of the structure-activity relationship analysis of the ligands, the following four conclusions can be drawn. First, for those ligands where the protonated nitrogen atom belongs to a planar conjugated system, i.e., quinazolines and AH11110A, and/or to a very rigid moiety, i.e., quinazolines, corynanthine, and spiperone, a strong inhibition of the constitutive activity of both α1-AR subtypes is observed. The quinazolines and corynanthine share a fixed distance between the protonated nitrogen and the center of its closest aromatic ring of ~2.8 and 3.4 Å, respectively (Fig. 5). Also the angle between the plane of the charge reinforced hydrogen bond and that of its closest aromatic ring is fixed in these compounds.

Second, for those ligands where the protonated nitrogen is almost in the middle of a flexible alkylic chain and the positive charge is almost equally distributed on two amine hydrogens, i.e., BE 2254, tamsulosin and RS-17053, the basal activity of the α_{1a}-AR is only partially inhibited. Due to the flexibility of this class of compounds, the distance between the protonated nitrogen and the center of its closest aromatic ring may vary, reaching a maximum of >5 Å (Fig. 5). Thus, the peculiar functional behavior of the phenylalkylamines may be related, at least in part, to the fact that the charge-reinforced hydrogen-bonding interaction with the receptor can occur through either one or both amine protons. Thus, different reciprocal orientations and distances between the charge-reinforced hydrogen bond and its closest aromatic ring are allowed during interaction with the receptor. The more pronounced inverse agonism of WB 4101 with respect to the other phenylalkylamines may be partially due to the fact that one of the two methylenic groups that separates the protonated nitrogen atom from its closest aromatic moiety belongs to a cycle, i.e., dioxane. This peculiarity may reduce the degrees of freedom of the molecule in proximity of the protonated nitrogen, decreasing the number of the allowed interacting modes with the receptor.

Third, the *N*-arylpiperazines show neutral antagonism at the α_{1a}-AR, with the exception of WAY 100635. This class of compounds is characterized by a fixed distance between the protonated nitrogen and the center of its closest aromatic ring of ~5.7 Å (Fig. 5). Moreover, the angle between the plane of the charge reinforced hydrogen bond and the plane of its closest aromatic ring may vary, at least to a small extent, in these compounds. The different behavior of WAY 100635 may be due to the topology of the carbonylic oxygen and of the pyridinic nitrogen. Conformational analysis showed that either one of these two heteroatoms may alternately perform intramolecular hydrogen bonds with the protonated nitrogen, thus stabilizing the different local minima (results not shown). These peculiar conformational properties may influence the docking mode of this ligand.

Forth, in *S*-(+)-niguldipine and SNAP 5089, the protonated nitrogen is in proximity of two aromatic rings that lie in position 4 of the piperidinic ring. In these compounds, the distance between the protonated nitrogen and the center of the aromatic rings in the equatorial and axial position 4 are ~5.9 and 4.6 Å, respectively (Fig. 5). Probably, the phenyl ring in the axial position is partially responsible of the inhibitory effect of these two compounds. Differently from all the other compounds considered in this study, the inverse agonism of *S*-(+)-niguldipine and SNAP 5089 at the α_{1b}-AR is less pronounced than at the α_{1a}-AR.

In summary, the results of this preliminary structure-activity relationship analysis suggest that the constitutive activity of the α_{1a}-AR can be differently inhibited by the tested ligands in a manner that seems dependent on the structural feature of the protonated nitrogen of the ligand and its distance from the closest aromatic moiety. However,

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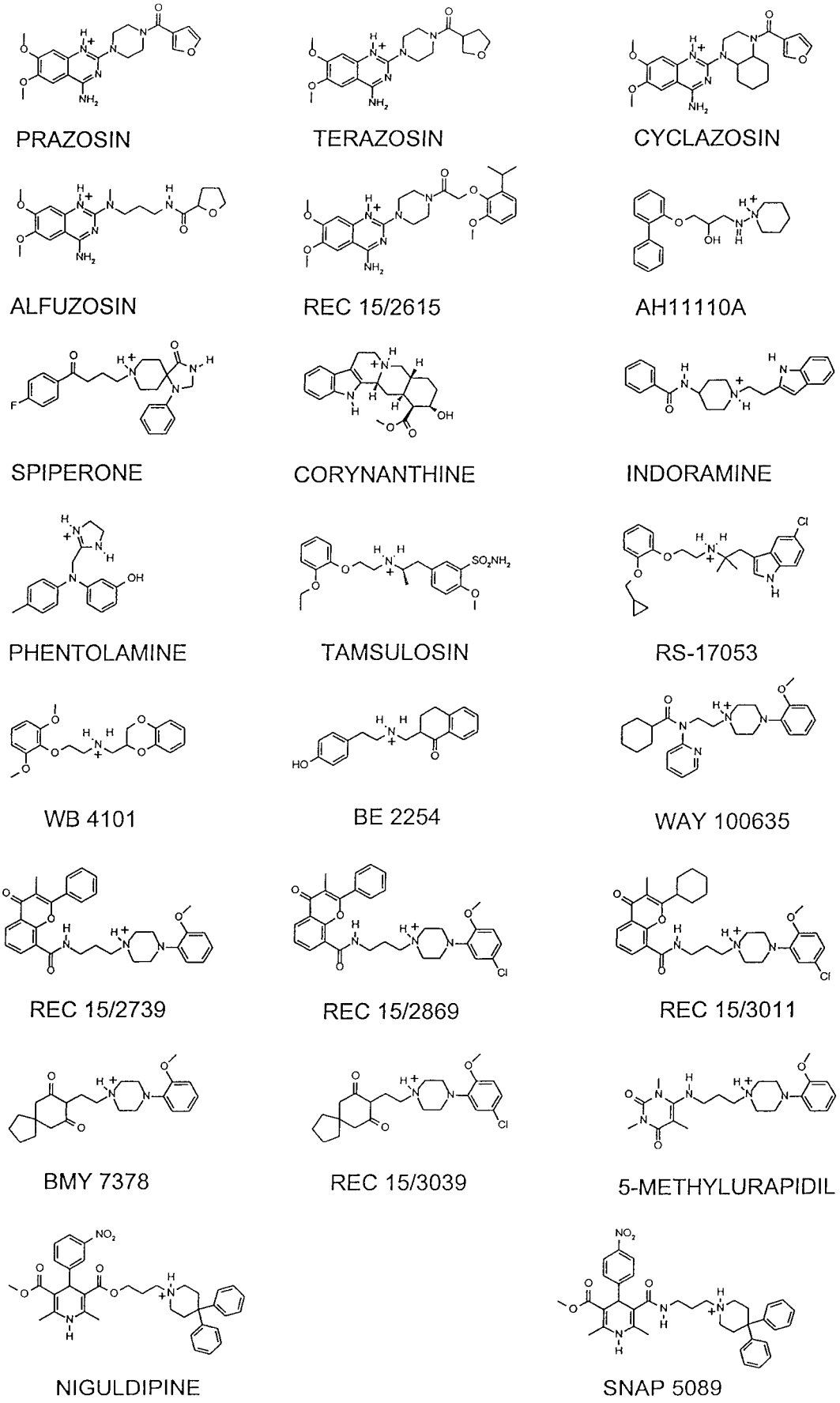

Fig. 4.

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a more accurate structure-activity relationship analysis-would require testing a much larger number of compounds for each structural group as well as the identification of the docking sites of the α_{1a}-AR and α_{1b}-AR for the various ligands.

Ligand Affinities at Wild-Type and CAM Receptors. The allosteric ternary complex model of receptor activation (Samama et al., 1993a) predicts that the transition from the R to R* states of the receptor can be influenced by ligand binding. The allosteric effect exerted by the ligand on the equilibrium between R and R* is given by the parameter β, which is related to the ligand efficacy. Whereas neutral antagonists ($\beta = 1$) have no effect on this equilibrium, agonists ($\beta > 1$) and inverse agonists ($\beta < 1$) will preferentially bind to R* and R, respectively. Thus, activating mutations, which are suggested to increase the stability of J for the conversion of R to R*, also will change ligand binding affinity. CAMs are predicted to display increased affinity for agonists and decreased affinity for inverse agonists compared with wild-type GPCRs, whereas the affinity of neutral antagonists should be similar (Samama et al., 1993b).

To test this hypothesis, the affinity of various ligands characterized by different efficacies was measured in membranes derived from COS-7 cells expressing the wild-type α_{1a}- and α_{1b}-AR or their mutants A271E and A293E, respectively. Because the unlabeled form of [^{125}I]HEAT is a partial inverse agonist (indicated as BE 2254 in Fig. 2) and that of [^{3}H]prazosin is a full inverse agonist at both receptor subtypes, the affinities of different ligands were measured with both radioligands.

As shown in Table 1, the K_d values of [^{125}I]HEAT and [^{3}H]prazosin were not significantly different at the wild-type α_{1a}- and α_{1b}-AR versus their CAMs. The results of competition binding experiments with [^{125}I]HEAT indicated that the K_i values of several α-blockers were not significantly different at the A271E or A293E mutants compared with their respective wild-type receptors (Table 3). Similar findings were obtained when the K_i values were measured with [^{3}H]prazosin as radioligand or with tracer concentrations (~10 pM) of [^{125}I]HEAT (results not shown). In conclusion, our results indicate that α-blockers with different negative efficacies do not display significantly different binding affin-

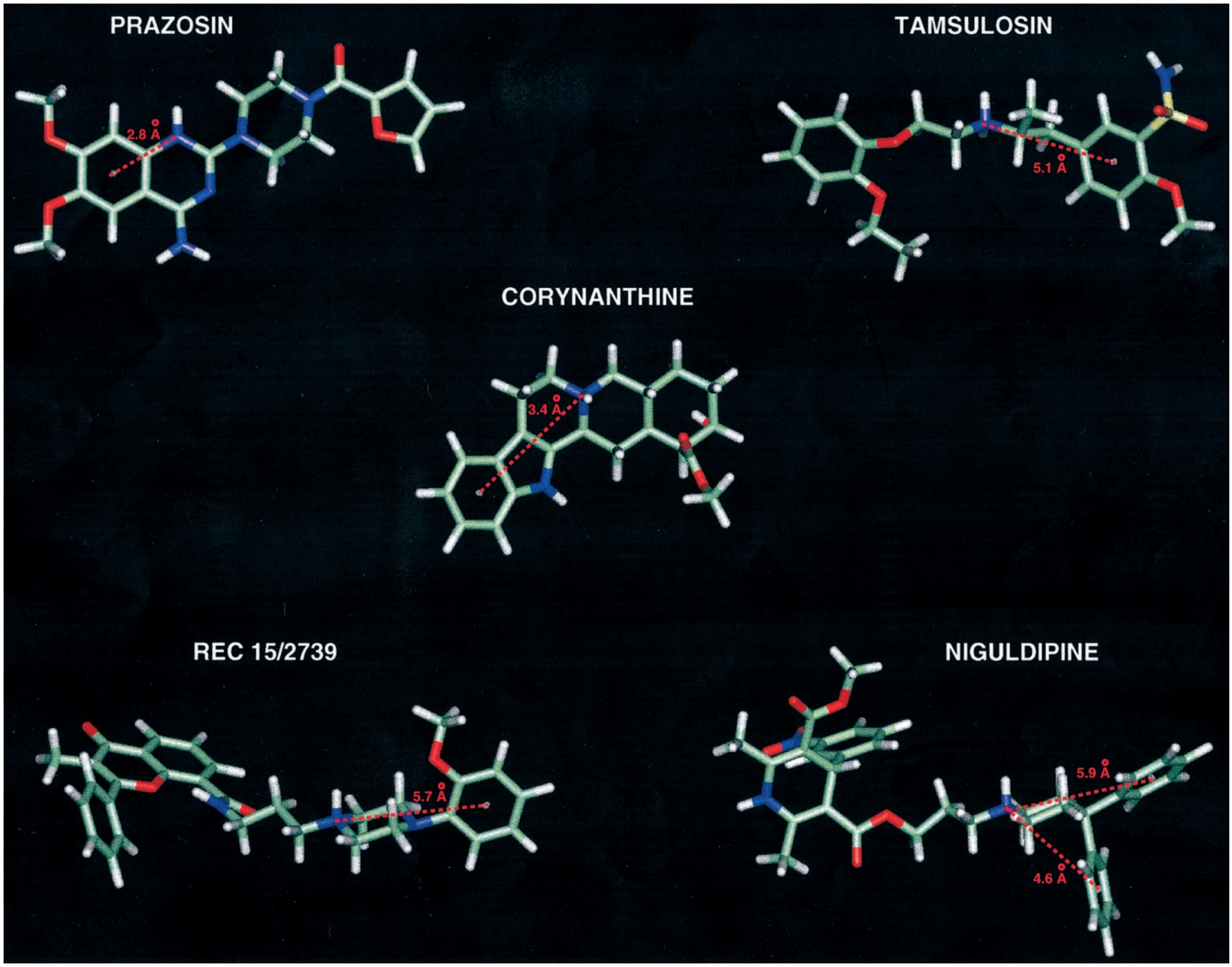

Fig. 5. Molecular models of some α-blockers. Molecular modeling of the ligands was performed as described in *Experimental Procedures*. The distances between the protonated nitrogen atom and its closest aromatic moiety are shown for each ligand.

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ities for the constitutively active forms of the α_{1a}- and α_{1b}-AR subtypes.

In contrast, the affinity of the full agonist epinephrine was increased 10-fold for A271E and 30-fold for A293E compared with that of their respective wild-type receptors. K_i values of epinephrine were 5.5 and 2.5 μM for the α_{1a}- and α_{1b}-AR, and 0.5 and 0.09 μM for A271E and A293E, respectively. The results are the mean of three to six independent determinations that did not differ by >40%).

These results can find their interpretation in the framework of the allosteric ternary complex model (Samama et al., 1993b). As predicted by the model, activating mutations of both the α_{1a}- and α_{1b}-AR result in a large increase in affinity for the full agonist epinephrine. However, changes in affinity for ligands with different efficacy (defined by the β parameter) depend on the level of receptor isomerization (defined by the J parameter). As demonstrated by the relationship between the difference in ligand affinity between the CAM versus wild-type receptor and the isomerization constant J of the receptor (Samama et al., 1993b), the mutation-induced change of affinity can be much larger for full agonists ($\beta > 1$) than for inverse agonists ($\beta < 1$), depending on the value of J. For example, the affinity of isoproterenol for a constitutively active mutant of the β_2-AR was 25-fold higher, whereas that of a full inverse agonist was 2-fold lower than for the wild-type receptor (Samama et al., 1993b).

Computer simulations predict that if an activating mutation increases the value of J by two orders of magnitude from 0.001 to 0.1, it can increase the affinity of a full agonist (β = 1000) 100-fold without changing the affinity of inverse agonists (Samama et al., 1993b). In this context, our findings suggest that wild-type α_{1a}-AR and α_{1b}-AR might be characterized by a very low isomerization level that is increased by the activating mutations of A271 or A293 into glutamic acid, respectively. This is consistent with the low spontaneous activity of both wild-type receptors. However, the mutation-induced increase in the isomerization constant J might not be sufficiently large to decrease the affinity of the CAMs for the inverse agonists.

Our findings are also in agreement with those from a previous report showing that the constitutive activation of the α_{1b}-AR resulting from a single-residue mutation is not sufficient to decrease the binding affinity of prazosin (Hwa et al., 1997). A decreased affinity for prazosin was observed only when multiple activating mutations were combined in the α_{1b}-AR.

Conclusions. Our findings suggest that CAMs carrying mutations at the C-terminal end of the 3i loop represent a useful tool to identify drugs that can behave as inverse agonists at wild-type α_{1a}- and α_{1b}-AR subtypes. This also might be generalized to other GPCRs because mutations at the carboxyl end of the 3i loop might alter the isomerization of the receptor to its active forms without directly interfering with the docking process of the ligands. However, our findings demonstrated that some partial inverse agonists displayed more pronounced negative efficacy at wild-type receptors than at their CAMs. This observation might be helpful to compare results from studies in which inverse agonism has been investigated in different experimental systems, i.e., on wild-type receptors versus their CAMs.

The results of our study demonstrate that a large number of structurally different α-blockers, including all the tested quinazolines are inverse agonists at both the α_{1a}- and α_{1b}-AR subtypes. In contrast, several *N*-arylpiperazines displayed different properties at the two α1-AR subtypes being inverse agonists with profound negative efficacy at the α_{1b}-AR, but not at the α_{1a}-AR. An important finding of our study is that REC 15/3039, REC 15/2739, and REC 15/3011 are the first α-blockers identified so far that do not display inverse agonism at one of the α1-AR subtypes, namely, α_{1a}.

The quinazolines, including prazosin, terazosin, and alfuzosin, which are among the most commonly used α-blockers, can display unwanted effects in vivo on the cardiovascular system such as orthostatic hypotension. In contrast, REC 15/2739, REC 15/2869, and REC 15/3011 are characterized by high selectivity for the urogenital tissues and seem to have less pronounced effects on the cardiovascular system in vivo (Testa et al., 1997). This was mainly demonstrated by the fact that in anesthetized dogs at doses effective in inhibiting norepinephrine-induced urethral contractions prazosin resulted in a decrease in dyastolic blood pressure, whereas the REC compounds did not. The pharmacological effects of REC 15/3039 in vivo or in different tissues have not been investigated. In future studies, it will be interesting to identify novel α-blockers that are neutral antagonists at one or more α1-AR subtypes and to assess whether these compounds, including REC 3039, have fewer generalized cardiovascular effects compared with full inverse agonists.

Inverse agonists of GPCRs might be of therapeutic use in pathological conditions resulting from activating mutations of receptors (Milligan et al., 1995). However, in most cases drugs are used to limit the action of endogenous agonists and inverse agonists should have no benefit over neutral antagonists. The clinical effect of inverse agonists could be relevant in tissues where GPCRs display high constitutive activity. Unfortunately, because spontaneous activity of wild-type receptors in vivo is difficult to assess, the clinical implications for the use of inverse agonists versus neutral antagonists remain a matter of debate. However, the distinction of receptor blockers as inverse agonists versus neutral antagonists might allow a better interpretation of the pharmacological effects of clinically used drugs with respect to their mechanism of action and contribute to assessing the therapeutic implications of inverse agonism.

The results of our work might provide an important contribution to further elucidating the pharmacological effects of drugs acting at the α1-AR subtypes and to optimizing their therapeutic use. They also provide useful information about the structure-activity relationships of α-blockers. In the future, mutagenesis studies aimed at identifying the docking sites on the α1-AR subtypes for inverse agonists and neutral antagonists might help in delineating receptor domains crucially involved in the inhibition of receptor isomerization and activation.

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Send reprint requests to: Susanna Cotecchia, M.D., Institut de Pharmacologie et de Toxicologie, 27, Rue du Bugnon, Faculté de Médecine, 1005 Lausanne, Switzerland. E-mail: susanna.cotecchia@ipharm.unil.ch

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