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Direct Evidence Revealing Structural Elements Essential for the High Binding Ability of Bisphenol A to Human Estrogen-Related Receptor-γ

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Background: Various lines of evidence have shown that bisphenol A [BPA; HO-C_6H_4-$C(CH_3)_2$-C_6H_4-OH] acts as an endocrine disruptor when present in very low doses. We have recently demonstrated that BPA binds strongly to human estrogen-related receptor-γ (ERR-γ) in a binding assay using [^{3}H]4-hydroxytamoxifen ([^{3}H]4-OHT). We also demonstrated that BPA inhibits the deactivation activity of 4-OHT.

Objectives: In the present study, we intended to obtain direct evidence that BPA interacts with ERR-γ as a strong binder, and also to clarify the structural requirements of BPA for its binding to ERR-γ.

Methods: We examined [^{3}H]BPA in the saturation binding assay using the ligand binding domain of ERR-γ and analyzed the result using Scatchard plot analysis. A number of BPA derivatives were tested in the competitive binding assay using [^{3}H]BPA as a tracer and in the luciferase reporter gene assay.

Results: [^{3}H]BPA showed a K_D of 5.50 nM at a B_{max} of 14.4 nmol/mg. When we examined BPA derivatives to evaluate the structural essentials required for the binding of BPA to ERR-γ, we found that only one of the two phenol-hydroxyl groups was essential for the full binding. The maximal activity was attained when one of the methyl groups was removed. All of the potent BPA derivatives retained a high constitutive basal activity of ERR-γ in the luciferase reporter gene assay and exhibited a distinct inhibitory activity against 4-OHT.

Conclusion: These results indicate that the phenol derivatives are potent candidates for the endocrine disruptor that binds to ERR-γ.

Key words: bisphenol A, constitutive activity, endocrine disruptor, estrogen receptor, estrogen-related receptor-γ, inverse agonist, nuclear receptor. *Environ Health Perspect* 116:32–38 (2008). doi:10.1289/ehp.10587 available via *http://dx.doi.org/* [Online 5 October 2007]

Bisphenol A [BPA; 2,2-bis(4-hydroxyphenyl)propane] has a symmetrical chemical structure of HO-C_6H_4-$C(CH_3)_2$-C_6H_4-OH. BPA is used mainly in the production of polycarbonate plastics and epoxy resins. Its worldwide manufacture is approximately 3.2 million metric tons/year. BPA has been acknowledged to be an estrogenic chemical able to interact with human estrogen receptors (ER) (Dodds and Lawson 1938; Krishnan et al. 1993; Olea et al. 1996), and many lines of evidence have revealed that BPA, at even low doses, acts as an endocrine disruptor (Gupta 2000; Nagel et al. 1997; vom Saal et al. 1998; Welshons et al. 2003). However, its binding to and hormonal interaction with ER are extremely weak, 2–3 orders of magnitude lower than those of natural hormones, and thus the intrinsic significance of these low-dose effects is rather intangible and obscure (Safe et al. 2002). These facts led us to hypothesize that BPA may interact with nuclear receptors (NRs) other than ER.

We have recently demonstrated that BPA binds strongly to estrogen-related receptor-γ (ERR-γ) with high constitutive activity (Takayanagi et al. 2006). ERR-γ is a member of the human NR family and the estrogen-related receptor (ERR) subfamily of orphan NRs, which are closely related to the ERs ER-α and ER-β (Giguère 2002; Horard and Vanacker 2003). The ERR family includes three members—ERR-α, ERR-β, and ERR-γ—with ERR-γ being the most recently identified (Eudy et al. 1998; Hong et al. 1999). The amino acid sequences are quite highly conserved among ERRs and ERs, but 17β-estradiol (E_2), a natural ligand of ERs, does not bind to any of the ERR family members. Our discovery that BPA binds strongly to ERR-γ, but not to ERs, indicates that the effects of the so-called endocrine disruptors should be examined for all NRs without delay.

ERR-γ is expressed in a tissue-restricted manner—for example, very strongly in the mammalian brain during development, and then in the brain, lung, and many other tissues during adulthood (Eudy et al. 1998; Heard et al. 2000; Lorke et al. 2000). Our preliminary results have shown that the highest expression is brought about in the placenta (Takeda Y, Sumiyoshi M, Liu X, Matsushima A, Shimohigashi M, Shimohigashi Y, unpublished data). Strong binding of BPA to ERR-γ would affect not only the physiologic functions but also the metabolism of this NR as a transcription-activating factor. Although the intrinsic physiologic functions of ERR-γ have not yet been clarified, it is crucial that a structure–function study be performed to clarify the structural requirements for the binding of BPA to ERR-γ.

In a previous study (Takayanagi et al. 2006), we used tritium (^{3}H)-labeled 4-hydroxytamoxifen (4-OHT) as a tracer in a receptor binding assay for ERR-γ. 4-OHT binds strongly to ERR-γ and deactivates it as an inverse agonist, decreasing the very high level of spontaneous constitutive activity (Coward et al. 2001). As a substitute for [^{3}H]4-OHT, BPA was found to be as potent as 4-OHT in this binding assay. Furthermore, BPA was found to retain or rescue ERR-γ's high basal constitutive activity in the reporter gene assay for ERR-γ using HeLa cells. These results indicated that BPA and 4-OHT bind to ERR-γ with equal strength, but have structural differences that affect their occupation of ERR-γ's ligand binding pocket. In the complex formed between 4-OHT and the ERR-γ–ligand binding domain (LBD), 4-OHT remained at the ligand binding pocket of ERR-γ–LBD, but the α-helix 12 of the receptor was repositioned from the activation conformation (Greschik et al. 2004; Wang et al. 2006). In contrast, BPA was suggested to bind to the pocket without changing the positioning of helix 12, and thus preserved the high receptor constitutive activity of ERR-γ.

It is evident that the binding ability of BPA to ERR-γ should be examined by means of tritium-labeled BPA. Fortunately, [^{3}H]BPA is now commercially available; thus, in the present study we performed the first saturation binding assay for direct exploration of the binding characteristics of BPA. We then established a competitive receptor binding assay in which chemicals were assessed for their ability to displace [^{3}H]BPA from the receptor binding pocket. In particular, industrial chemical products of BPA analogs were inspected structurally in order to better understand the

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structural elements of BPA that are required for binding to the ERR. Here we describe the structural elements of BPA that are required for the binding to ERR-γ–LBD and for maintaining the receptor in an active conformation.

Materials and Methods

Chemicals. We purchased 2,2-bis(4-hydroxyphenyl)propane and 4,4-isopropylidenediphenol, both denoted as BPA, from Tokyo Kasei Kogyo Co. (Tokyo, Japan), Nakarai Tesque (Kyoto, Japan), Aldrich (Madison, WI, USA), Junsei Chemical (Tokyo, Japan), Acros (Geel, Belgium), Lancaster Synthesis (Windham, NH, USA), Merck (Darmstadt, Germany), and Fluka (Buchs, Switzerland). The purity designated on the labels varied from 95 to 99%. We also obtained the following analogs of BPA: bisphenol AF [2,2-bis(4-hydroxyphenyl)hexafluoropropane; Tokyo Kasei], bisphenol AP [4,4′-(1-phenylethylidene)bisphenol; Tokyo Kasei], bisphenol B [2,2-bis(4-hydroxyphenyl)butane; Tokyo Kasei], bisphenol E [2,2-bis(4-hydroxyphenyl)ethane; Aldrich], and bisphenol F [bis(4-hydroxyphenyl)methane; Tokyo Kasei].

4-α-Cumylphenol [2-(4-hydroxyphenyl)-2-phenylpropane], 4-*tert*-amylphenol, 4-*tert*-butylphenol, 4-isopropylphenol, and 4-ethylphenol were obtained from Tokyo Kasei. 2,2-Diphenyl propane, and 4-*tert*-octylphenol were obtained from Aldrich, and *p*-cresol and phenol from Kishida Chemical (Osaka, Japan).

Preparation of receptor protein GST-fused ERR-γ–LBD. ERR-γ–LBD was amplified from a human kidney cDNA library (Clontech Laboratories, Mountain View, CA, USA) by polymerase chain reaction (PCR) using gene-specific primers and cloned into pGEX6P-1 (Amersham Biosciences, Piscataway, NJ, USA). Glutathione *S*-transferase (GST)–fused receptor protein expressed in *Escherichia coli* BL21α was purified on an affinity column of glutathione-sepharose 4B (GE Healthcare Bio-Sciences Co., Piscataway, NJ, USA) to obtain GST-ERR-γ–LBD. The glutathione used for elution of GST-ERR-γ–LBD from the column was removed by gel filtration on a column of Sephadex G-10 (15 × 100 mm; GE Healthcare Bio-Sciences Co.) equilibrated with 50 mM Tris-HCl (pH 8.0), and the protein content (506.24 μg/mL) was estimated by the Bradford method using a Protein Assay CBB Solution (Nakarai Tesque). Preparation of GST-fused ER-α–LBD was carried out as described previously (Takayanagi et al. 2006).

Radioligand binding assays for saturation binding. The saturation binding assay for GST-ERR-γ–LBD was conducted at 4°C using [^{3}H]BPA (5 Ci/mmol; Moravek Biochemicals, Brea, CA, USA) with or without BPA (10 μM in the final solution). Purified protein (0.32 μg/mL) was incubated with increasing concentrations of [^{3}H]BPA (2.1–24.3 nM) in a final volume of 100 μL of binding buffer [10 mM HEPES (pH 7.5), 50 mM sodium chloride, 2 mM magnesium chloride, 1 mM EDTA, 2 mM CHAPS {3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate}, and 2 mg/mL γ-globulin]. Nonspecific binding was determined in a parallel set of incubations that included 10 μM nonradiolabeled BPA. After incubation for 2 hr at 4°C, all the fractions were filtered by the direct vacuum filtration method (MultiScreen$_{HTS}$ HV, 0.45 μm pore size; Millipore, Billerica, MA, USA) for the B/F separation (the separation of receptor-bound ligand from free ligand) (Nakai et al. 1999). Filtration was carried out on a multiscreen separation system (Millipore). Before filtration, 100 μL of 1% dextran-coated charcoal (DCC) (Sigma) in phosphate buffer (pH 7.4) was added to the assay vessels, and the mixture was incubated for 10 min on ice. The radioactivity of the filtered solution was counted on a liquid scintillation counter (LS6500; Beckman Coulter, Fullerton, CA, USA). The saturation assay was performed in triplicate. The specific binding of [^{3}H]BPA was calculated by subtracting the nonspecific binding from the total binding.

Radioligand binding assays for competitive binding. BPA and the BPA-related chemicals were dissolved in a binding buffer containing 0.3–1.0% *N,N*-dimethylsulfoxide (DMSO). These compounds were examined for their ability to inhibit the binding of [^{3}H]BPA (3 nM in the final solution) to GST-ERR-γ–LBD (0.32 μg/mL in the final solution). The reaction mixtures were incubated for 2 hr at 4°C and free radioligand was removed with 1% DCC by filtration as described above. Radioactivity was determined on a liquid scintillation counter (TopCount NXT; PerkinElmer Life Sciences Tokyo, Japan). The IC_{50} values (the concentrations for the half-maximal inhibition) were calculated from the dose–response curves obtained using the nonlinear analysis program ALLFIT (De Lean et al. 1978). Each assay was performed in duplicate and repeated at least three times. The competitive binding assay for GST-ER-α–LBD was carried out as described above using [^{3}H]E_2 (5.74 TBq/mmol; Amersham Biosciences, Buckinghamshire, UK).

Cell culture and transient transfection assays. HeLa cells were maintained in Eagle's MEM (EMEM; Nissui, Tokyo, Japan) in the presence of 10% (vol/vol) fetal bovine serum at 37°C. For luciferase assays, HeLa cells were seeded at 5 × 10^5 cells/6-cm dish for 24 hr and then transfected with 4 μg of reporter gene (pGL3/3×ERRE) and 3 μg of ERR-γ expression plasmids (pcDNA3/ERR-γ) by Lipofectamine Plus reagent (Invitrogen Japan, Tokyo, Japan) according to the manufacturer's protocol. Approximately 24 hr after transfection, cells were harvested and plated into 96-well plates at 5 × 10^4 cells/well. The cells were then treated with varying doses of chemicals diluted with 1% bovine serum albumin/phosphate-buffered saline (BSA/PBS, vol/vol). To measure the antagonistic activity, a fixed concentration of compounds (10^{-5} M to 10^{-10} M in the final solution) was added along with 4-OHT. After 24 hr, luciferase activity was measured with the appropriate reagent using a Luciferase Assay System (Promega, Madison, WI, USA) according to the manufacturer's instructions. Light emission was measured using a Wallac 1420 ARVOsx multilabel counter (PerkinElmer). Cells treated with 1% BSA/PBS were used as a vehicle control. Each assay was performed in triplicate and repeated at least three times.

Results and Discussion

Highly specific binding of BPA to ERR-γ. To demonstrate the direct binding of BPA to ERR-γ, we first attempted to establish a saturation receptor binding assay using radiolabeled BPA. We analyzed the saturation binding of [^{3}H]BPA against the recombinant ERR-γ–LBD protein, to which GST was fused at the N-terminus. In the actual receptor binding assay, we used [^{3}H]BPA (2.0–24 nM) against purified protein at a concentration of 0.32 μg/mL, which corresponds to a concentration of 6.3 nM. The removal of receptor-free [^{3}H]BPA was carried out with 1% DCC. In this procedure, DCC mixtures were transferred to a 96-well HV-plate with a filter (0.45-μm pore size) for direct vacuum.

As shown in Figure 1A, the binding of BPA to ERR-γ was specific and saturated. Specific binding of [^{3}H]BPA to ERR-γ was estimated to be approximately 80%, which we judged to be a very high value. In other words, the level of nonspecific binding of [^{3}H]BPA was very low (Figure 1A). The high level of specific binding of [^{3}H]BPA clearly demonstrated that BPA has no structural elements for nonspecific binding to the receptor protein and exclusively occupies the binding pocket of ERR-γ–LBD. GST did not bind [^{3}H]BPA at all. It should be noted that the specific binding of [^{3}H]4-OHT was only about 50% (Takayanagi et al. 2006).

The Scatchard plot analysis showed a distinct single binding mode (Figure 1B). From the slope, the binding affinity constant (K_D) was calculated to be 5.50 nM. The receptor density (B_{max}) was estimated to be 14.4 nmol/mg protein, which is roughly compatible with the calculated value of 18.9 nmol/mg protein. The B_{max} value of [^{3}H]4-OHT is much smaller than that of [^{3}H]BPA. These results further demonstrate that ERR-γ binds [^{3}H]BPA very specifically and exclusively.

Binding ability of BPA to ERR-γ. We performed the competitive receptor binding assay using [^{3}H]BPA (3 nM in the final solution) for GST–ERR-γ–LBD (0.32 μg/mL in the final solution). To confirm that BPA is a truly specific ligand for ERR-γ, we tested all nonradiolabeled BPA compounds available in Japan, which we obtained from seven different reagent companies. Because the compounds all had different levels of purity (95–99%), we adjusted their initial concentration, 1.0×10^{-2} M, based on the purity indicated on the label.

We found that BPA displaces [^{3}H]BPA in a dose-dependent manner. Its binding curve was sigmoidal in a single binding mode (slope = ~ 1), which afforded an average IC_{50} value of 9.78 nM. We found all BPA compounds purchased to be equally potent. These results clearly demonstrate that BPA binds very strongly to the NR ERR-γ.

4-OHT as a potent displacer of BPA in ERR-γ. 4-OHT has been reported to potently displace [^{3}H]4-OHT in the binding to ERR-γ (Greschik et al. 2004; Takayanagi et al. 2006). In the present study, 4-OHT very potently displaced [^{3}H]BPA (IC_{50} = 10.9 nM) (Table 1). BPA and 4-OHT yielded sigmoidal binding curves indistinguishable from each other (data not shown), indicating that the two are almost equipotent. These results obtained using the [^{3}H]BPA tracer were almost identical to those obtained by [^{3}H]4-OHT (Takayanagi et al. 2006).

BPA and 4-OHT share only a phenol group, and thus the phenol groups of these compounds are highly likely to occupy the same binding site in the ERR-γ receptor. Because the phenol group of 4-OHT is anchored by hydrogen bonds to Glu275 and Arg316 of ERR-γ (Greschik et al. 2004), the phenol group of BPA may also bind to these ERR-γ residues. Indeed, this has been proven by our recent X-ray crystal structure analysis of the complex between BPA and human ERR-γ–LBD (Matsushima et al. 2007). Hereafter, we designate the benzene ring of this phenol group of BPA as the A-ring and the additional benzene ring as the B-ring.

BPA-methyl as a structural requirement for binding to ERR-γ. We evaluated the role of the two methyl (CH_3) groups on the sp^3-C atom of BPA in binding to ERR-γ by a series of analogs of BPA, HO-C_6H_4-$C(CH_3)_2$-C_6H_4-OH. First, we examined the effect of incorporation of the methyl group on the binding affinity of BPA. When CH_3 was

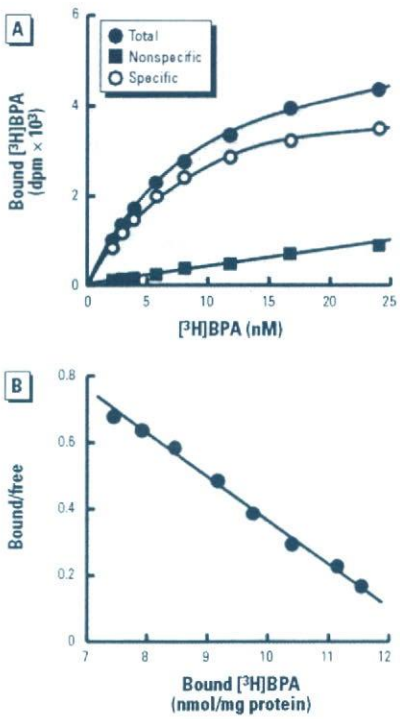

Figure 1. The saturation binding analysis of BPA for ERR-γ. (*A*) Saturation binding curve of [^{3}H]BPA for the recombinant human ERR-γ–LBD showing total, nonspecific, and specific binding. Determination of nonspecific binding was carried out by excess unlabeled BPA (10 μM). (*B*) Binding data analyzed by Scatchard plot analysis to estimate the dissociation constant (K_D) and the receptor density (B_{max}). The plot was linear, the K_D value was estimated to be 5.50 ± 0.87 nM, and B_{max} was 14.4 nmol/mg protein. The saturation binding analysis was performed in duplicate and repeated four times.

Table 1. Receptor binding affinity (mean ± SE) of BPA and its analogs, and 4-OHT for ERR-γ.

Chemical	Binding affinity (IC_{50}, nM)
BPA	9.78 ± 0.87
Bisphenol AF	358 ± 30.5
Bisphenol AP	123 ± 15.1
Bisphenol B	26.3 ± 2.65
Bisphenol E	8.14 ± 0.83
Bisphenol F	131 ± 17.9
4-OHT	10.9 ± 0.91

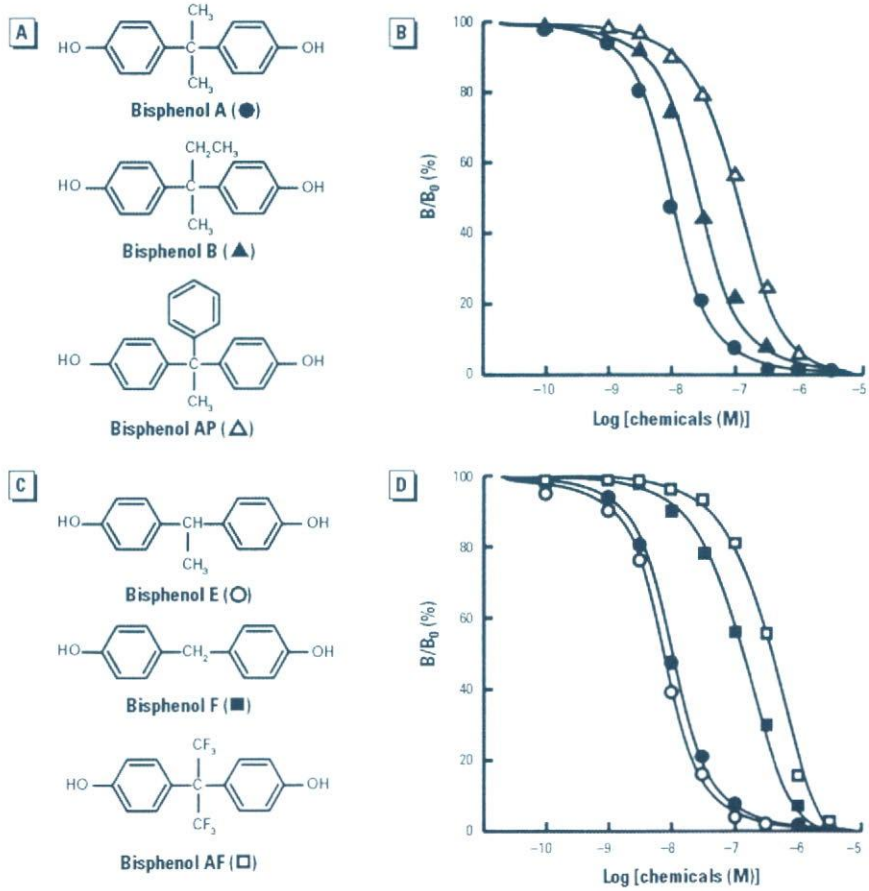

Figure 2. Chemical structure of BPA and its derivatives and their dose–response curves in the radioligand receptor binding assay for ERR-γ. (*A*) Chemical structures of BPA (two methyl groups) and its derivatives: bisphenol B (a methyl group and an ethyl group) and bisphenol AP (a methyl group and a phenyl group). (*B*) Binding activities of BPA, bisphenol B, and bisphenol AP examined by the competitive binding assay using [^{3}H]BPA and GST–ERR-γ–LBD. (*C*) Chemical structures of bisphenol E (one methyl group) and its derivatives, bisphenol F and bisphenol AF [two trifluoromethyl groups (CF_3)]. (*D*) Binding activities of BPA, bisphenol E, bisphenol F, and bisphenol AP examined by the competitive binding assay. (*B*) and (*D*) each show representative curves with the IC_{50} values closest to the mean IC_{50} from at least five independent assays for each compound. B/B_0 is the relative inhibitory activity estimated from the calculation of the percentage of displacement by the chemical tested (B) against the specific binding (B_0 = 100%) of [^{3}H]BPA.

incorporated into the parent methyl group to produce HO-C_6H_4-$C(CH_3)(CH_2CH_3)$-C_6H_4-OH (Figure 2A), we found the resulting bisphenol B to be approximately half as potent (IC_{50} = 26.3 nM) as BPA (Table 1). This result clearly indicates that a bulky group on the central sp^3-C atom is obviously disadvantageous in terms of the binding of BPA to ERR-γ's binding pocket.

On the other hand, an enhancement of activity was observed when one of the methyl groups was eliminated from BPA. The resulting bisphenol E [HO-C_6H_4-$CH(CH_3)$-C_6H_4-OH] (Figure 2C) exhibited slightly better binding activity (IC_{50} = 8.14 nM) than BPA (Table 1). Bisphenol E is indeed the most potent chemical to date for the NR ERR-γ (Figure 2D). The maximal activity was attained when one of the methyl groups was removed from BPA. Apparently, the concomitance of two methyl groups on the central sp^3-C atom of BPA is disadvantageous and unfavorable.

The fact that a single methyl group had the best fit for ERR-γ was further demonstrated by the diminished activity of bisphenol AP, which has a phenyl group in place of the hydrogen atom that is found in bisphenol E (Figure 2A). Bisphenol AP exhibited approximately 15-fold weaker binding affinity for ERR-γ than bisphenol E, with IC_{50} = 123 nM (Figure 2B, Table 1). Steric hindrance by the benzene ring, as well as its electron-rich characteristics, might be responsible for this drop in the receptor binding affinity of bisphenol AP.

The importance of the remaining methyl group in bisphenol E became evident from the drastically reduced activity of bisphenol F [HO-C_6H_4-CH_2-C_6H_4-OH]. This compound was approximately 16-fold less potent than bisphenol E, exhibiting an IC_{50} value of 131 nM (Table 1). All of these results clearly indicate that one of the two methyl groups is involved in the intermolecular interaction with the receptor residue(s). The interaction involving the CH_3 group is a kind of hydrophobic interaction, such as CH_3-alkyl and CH/π interactions.

The fundamental nature of this interaction involving the CH_3 group became rather apparent from the binding result of bisphenol AF [HO-C_6H_4-$C(CF_3)_2$-C_6H_4-OH]. The $CH_3 \rightarrow CF_3$ substitution in BPA creates this compound (Figure 2C), which has two electron-rich trifluoromethyl CF_3 groups instead of the rather electron-poor methyl CH_3 group. The molecular size of CF_3 is almost equal to that of CH_3. A drastically reduced activity of bisphenol AF, about 35-fold less potent (358 nM) than BPA (Table 1), thus demonstrates that the BPA's CH_3 group is in an electrostatic interaction with the electron-rich residue(s) of the receptor. Replacement of CH_3 with CF_3 is definitely disadvantageous, because CF_3 is very electron-rich and thus brings about a strong repulsion with such electron-rich residues of the receptor. One of the electron-rich candidates of the receptor is the aromatic ring of Phe, Tyr, His, and Trp. Based on the reported X-ray crystal structure of ERR-γ, feasible candidates are Phe-435 and Phe-450 (Greschik et al. 2002, 2004; Matsushima et al. 2007; Wang et al. 2006).

A single phenol-hydroxyl group is enough for BPA to bind to ERR-γ. BPA has a very simple symmetrical chemical structure of HO-C_6H_4-$C(CH_3)_2$-C_6H_4-OH (Figure 2A). When one of the phenol-hydroxyl groups (–OH) of BPA was eliminated, the resulting 4-α-cumylphenol (HO-C_6H_4-$C(CH_3)_2$-C_6H_5; Figure 3A) still bound very strongly to ERR-γ. 4-α-Cumylphenol was as potent as BPA (Figure 3B), having an IC_{50} value of 10.6 nM (Table 2). Contrary to the expectation that both of the phenol-hydroxyl groups of BPA would participate in the hydrogen bonds, this result indicates that the second hydroxyl group does not necessarily participate in the hydrogen bonding. Given that this hydroxyl group forms a hydrogen bond with the ERR-γ receptor residue, the bond would be considered extremely weak, as suggested by the X-ray crystal analysis of 4-α-cumylphenol–ERR-γ complex (Matsushima A, Teramoto T, Okada H, Liu X, Tokunaga T, Kakuta Y, Shimohigashi Y, unpublished data).

When both of the phenol-hydroxyl groups were eliminated from BPA, the resulting 2,2-diphenyl propane [C_6H_5-$C(CH_3)_2$-C_6H_5] was almost completely inactive (Figure 3B, Table 2). This compound elicits only about 30% inhibition of the binding of [^{3}H]BPA at the 1-μM concentration, whereas BPA almost completely inhibits the binding of [^{3}H]BPA at this concentration (Figure 3B). It is clear that one of the phenol-hydroxyl groups of BPA is indispensable for the interaction with a binding pocket of ERR-γ. These results, together with the fact that 4-α-cumylphenol and BPA are equipotent, emphasizes the significance of one of the two phenol groups in the interaction of BPA with ERR-γ. As described above, this hydroxyl group should be attached to the benzene A-ring. It became apparent that the phenol-hydroxyl group attached to another phenol-benzene ring (B-ring) is not necessarily required for binding of BPA to ERR-γ.

BPA-phenol as a structural requirement for binding to ERR-γ. As described above, 4-α-cumylphenol is as active as BPA. The importance of the benzene B-ring can be examined by replacing the B-ring with the alkyl groups. When the benzene B-ring of 4-α-cumylphenol was substituted with either methyl or ethyl, the resulting 4-*tert*-butylphenol [HO-C_6H_4-$C(CH_3)_2$-CH_3] and

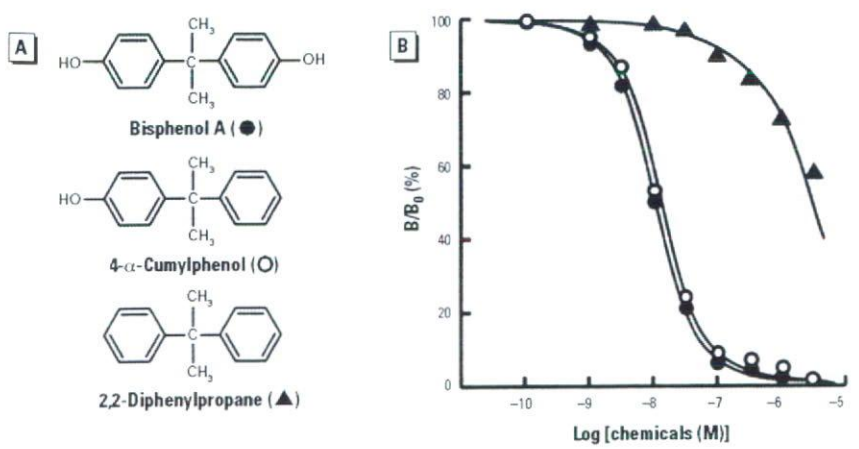

Figure 3. Chemical structure of BPA and its derivatives lacking the hydroxyl group(s) and their dose–response curves in the radioligand receptor binding assay for ERR-γ. (*A*) Chemical structure of BPA and its derivatives lacking the hydroxyl group(s): 4-α-cumylphenol (without one hydroxyl group from BPA), and 2,2-diphenylpropane (without either hydroxyl groups from BPA). (*B*) Binding activities of BPA, 4-α-cumylphenol, and 2,2-diphenylpropane examined by the competitive binding assay using [^{3}H]BPA and GST-ERR-γ–LBD; representative curves indicate the IC_{50} value closest to the mean IC_{50} from at least five independent assays for each compound.

Table 2. The receptor binding affinity (mean ± SE) of BPA and its derivatives lacking of the phenol group for human ERR-γ.

Chemical	Binding affinity (IC_{50}, nM)
BPA	9.78 ± 0.87
4-α-Cumylphenol	10.6 ± 0.87
2,2-Diphenylpropane	ND
4-*tert*-Butylphenol	26.1 ± 2.45
4-*tert*-Amylphenol	33.2 ± 2.85
4-Isopropylphenol	71.1 ± 7.73
4-*tert*-Octylphenol	238 ± 28.1
4-Ethylphenol	289 ± 45.9
p-Cresol	1,290 ± 72.5
Phenol	ND

ND, not determined (IC_{50} value could not be calculated because of extremely weak binding activity, even at a 10 μM concentration).

4-*tert*-amylphenol [HO-C_6H_4-$C(CH_3)_2$-CH_2CH_3] (Figure 4A) were considerably potent (Figure 4B), with values of 26.1 nM and 33.2 nM, respectively (Table 2). This reveals that alkyl groups can be substituted for the aromatic benzene ring without affecting the basal binding capability.

However, because both 4-*tert*-butylphenol and 4-*tert*-amylphenol are still a few times less active than 4-α-cumylphenol, a specific binding site of ERR-γ appears to prefer the aromatic benzene ring to the alkyl groups. This suggests that BPA's second phenol-phenyl group (benzene B-ring) is in the π interaction with the receptor residue(s), that is, either a XH/π interaction (X = N, O, and C) or a π/π interaction. The most plausible candidate for the receptor residue in this interaction is the Tyr residue at position 326 of ERR-γ. Indeed, the phenol-hydroxyl group of this Tyr-326 was found in the OH/π interaction with the B-ring of BPA (Matsushima et al. 2007).

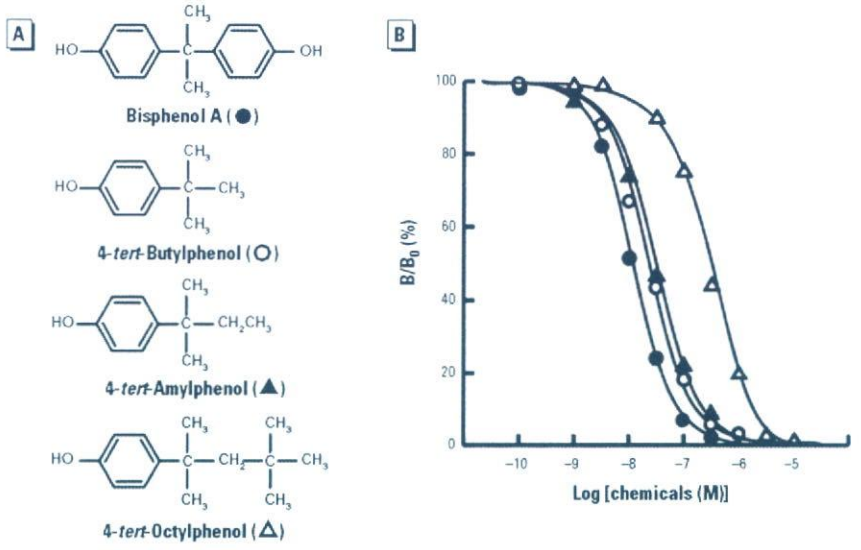

Figure 4. Chemical structure of BPA and its derivatives lacking the phenol group and their dose–response curves in the radioligand receptor binding assay for ERR-γ. (*A*) Chemical structure of BPA and its derivatives with the alkyl group at the position of phenol group: 4-*tert*-butylphenol (a methyl group); 4-*tert*-amylphenol (an ethyl group); and 4-*tert*-octylphenol (a *tert*-butyl methyl group). (*B*) Binding activities of BPA, 4-*tert*-butylphenol, 4-*tert*-amylphenol, and 4-*tert*-octylphenol examined by the competitive binding assay using [^{3}H]BPA and GST-ERR-γ–LBD; representative curves indicate the IC_{50} value closest to the mean IC_{50} from at least five independent assays for each compound.

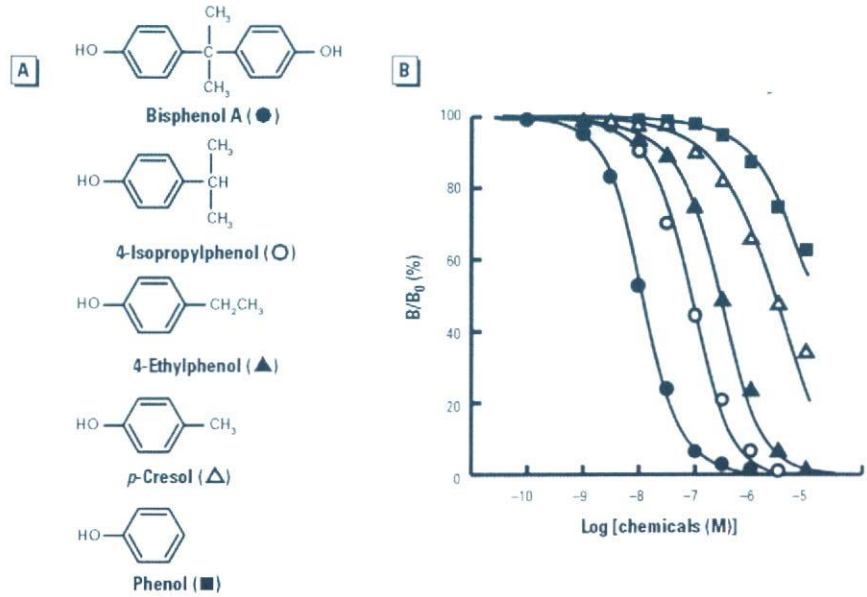

Figure 5. Chemical structure of BPA and a series of alkyl phenols and their dose–response curves in the radioligand receptor binding assay for ERR-γ. (*A*) Chemical structure of BPA and its derivatives with the alkyl group at the *para* position: 4-isopropylphenol (a 4-isopropyl group); 4-ethylphenol (an ethyl group); *p*-cresol (a methyl group); and phenol (a hydrogen atom). (*B*) Binding activities of BPA, 4-isopropylphenol, 4-ethylphenol, *p*-cresol, and phenol examined by the competitive binding assay using [^{3}H]BPA and GST-ERR-γ–LBD; representative curves indicate the IC_{50} value closest to the mean IC_{50} from at least five independent assays for each compound.

In a BPA molecule, two C_6H_4-OH (phenol) groups are connected to the sp^3 carbon atom (sp^3-C) together with two CH_3 (methyl) groups. The most simple structure–activity study is to compare the activity of compounds lacking one of these groups. The compound that lacks the phenol group is 4-isopropylphenol [HO-C_6H_4-$CH(CH_3)_2$] (Figure 5A), and this *para*-isopropyl phenol was fairly potent at displacing [^{3}H]BPA (Figure 5B), with an IC_{50} value of 71.1 nM (Table 2). However, 4-isopropylphenol was still approximately 7-fold less active than BPA, indicating that the phenol backbone structure is an essential structural element for the binding to ERR-γ.

When one of the two methyl groups was eliminated from 4-isopropylphenol, the resulting 4-ethylphenol [HO-C_6H_4-CH_2-CH_3] (Figure 5A) was found to be very weakly active (289 nM) (Table 2). Elimination of another methyl group still afforded a compound of inactive *p*-cresol [HO-C_6H_4-CH_3], but with the IC_{50} value being approximately 1.3 μM. Phenol [HO-C_6H_5] tended to bind to ERR-γ (Figure 5B). These results clearly indicate that the phenol group is a core structure for the attachment of BPA to ERR-γ.

4-Alkyl phenols as putative potent binders to ERR-γ. Attachment of the methyl group to 4-isopropylphenol [HO-C_6H_4-$CH(CH_3)_2$] to create 4-*tert*-butylphenol [HO-C_6H_4-$C(CH_3)_3$] considerably facilitates the binding of the phenol derivative to ERR-γ (Table 2). 4-*tert*-Amylphenol [HO-C_6H_4-$C(CH_3)_2$-CH_2CH_3] is almost as active as 4-*tert*-butylphenol. However, 4-*tert*-octylphenol [HO-C_6H_4-$C(CH_3)_2$-CH_2-$C(CH_3)_3$] (Figure 4B) was significantly weaker (approximately 10 times less potent) than 4-*tert*-butylphenol (Table 2). Thus, the activities of HO-C_6H_4-$C(CH_3)_2$-$CH(CH_3)_2$, HO-C_6H_4-$C(CH_3)_2$-$CH(CH_3)_3$, HO-C_6H_4-$C(CH_3)_2$-CH_2-CH_2-CH_3, and HO-C_6H_4-$C(CH_3)_2$-CH_2-$CH(CH_3)_2$ are expected to be intermediate between those of 4-*tert*-amylphenol and 4-*tert*-octylphenol, although these molecules are not commercially available. It appears that, among the 4-alkylphenols of HO-C_6H_4-$C(CH_3)_2$-C_nH_{2n+1} (=R), 4-*tert*-butylphenol (R = CH_3) and 4-*tert*-amylphenol (R = CH_2-CH_3) show the maximum competitive activity with the binding of ERR-γ.

The structural comparison of HO-C_6H_4-$C(CH_3)_2$-CH_3 (4-*tert*-butylphenol), HO-C_6H_4-$C(CH_3)_2$-CH_2CH_3 (4-*tert*-amylphenol),

and BPA HO-C_6H_4-$C(CH_3)_2$-C_6H_4-OH clearly indicated that the R group should not be bulky for high receptor binding activity. A plain π electron-rich benzene aromatic ring is thus optimal for interaction with the receptor residue of ERR-γ-Tyr326.

Inhibitory activity of BPA derivatives for ERR-γ. We found that BPA retained a high constitutive basal activity of ERR-γ in the luciferase reporter gene assay (Figure 6A). ERR-γ is in a full activation with no ligand; it is one of the self-activated NRs and is deactivated by the so-called "inverse agonists" such as 4-OHT (Greschik et al. 2004; Takayanagi et al. 2006). Although BPA shows no apparent effect on the high basal activity of ERR-γ, BPA evidently antagonizes or inhibits the deactivation activity of 4-OHT in a dose-dependent manner (Figure 6B), as reported by Takayanagi et al. (2006). This neutral antagonist is a distinct inhibitor or suppressor of the inverse agonist, reversing the deactivation conformation to the activation conformation.

All of the potent BPA derivatives (i.e., bisphenol E, bisphenol AF, 4-α-cumylphenol, and 4-*tert*-butylphenol) were found, just like BPA, to retain a high constitutive basal activity of ERR-γ in the same luciferase reporter gene assay (Figure 6C). In addition, these compounds inhibited the inverse agonist activity of 4-OHT and thus were specific inhibitors against the inverse agonist 4-OHT. Their abilities to antagonize 4-OHT are approximately one order lower than their binding potencies to ERR-γ (Figure 6B,D). This discrepancy is probably caused by the inclusion of a number of co-effecter proteins for eliciting a gene expression in the luciferase reporter gene assay.

Receptor selectivity of BPA derivatives for ERR-γ over ER-α. We classified BPA and its derivatives into the four groups, depending on their receptor binding affinity for ERR-γ: that is, group A, BPA and chemicals as potent as BPA; group B, chemicals considerably potent; group C, chemicals moderately potent; and group D, inactive chemicals. All chemicals were then examined for their ability to bind to ER-α, and the affinity measured was compared respectively with that for ERR-γ (Table 3). As reported previously (Takayanagi et al. 2006), BPA is highly selective for ERR-γ. It binds to ER-α only weakly; we calculated BPA's receptor selectivity to be 105, which suggests that BPA prefers ERR-γ 105 times more strongly than ER-α. Other group A compounds, namely, bisphenol E and 4-α-cumylphenol, were also greatly selective for ERR-γ (Table 3). In particular, bisphenol E was found to be exclusively selective and specific for ERR-γ because it was almost completely inactive for ER-α.

para-Alkyl phenols in group B ($IC_{50}^{ERR\text{-}\gamma}$ = of 26–71 nM) were also almost completely inactive for ER-α (Table 3). Those include

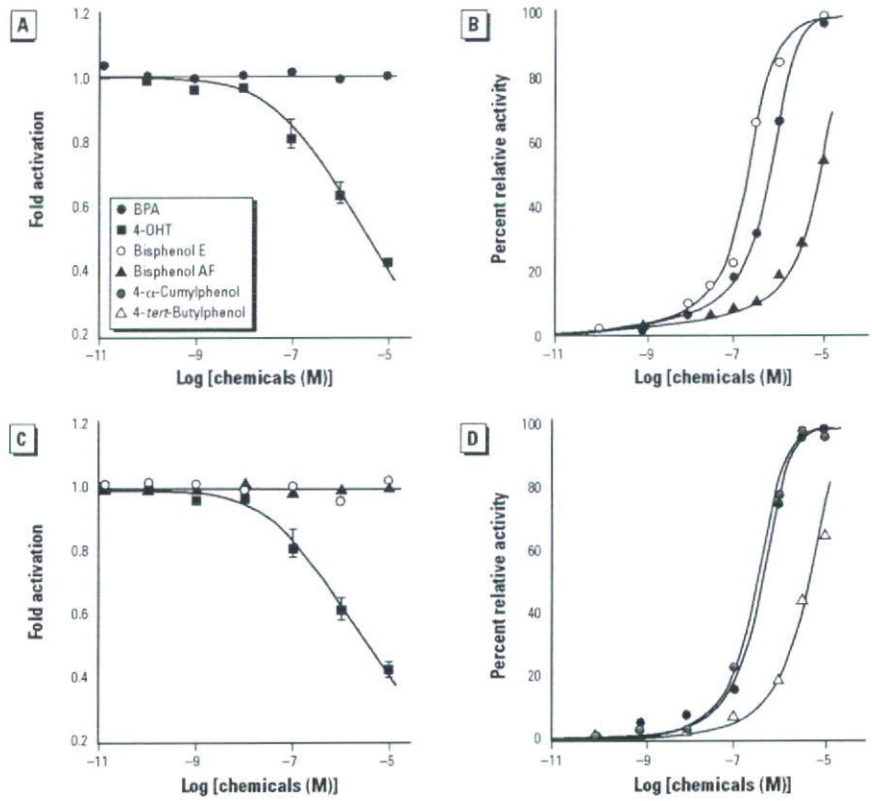

Figure 6. Luciferase-reporter gene assay of BPA and its derivatives for human ERR-γ. (*A*) Deactivation of the fully activated human ERR-γ by the inverse agonist 4-OHT and sustainment by BPA. (*B*) Reversing activity of BPA, bisphenol E, and bisphenol AF against the inverse agonist activity of 1.0 μM 4-OHT; 1.0 μM 4-OHT exhibited approximately 0.4-fold deactivation, and the inhibitory activities are shown by the percentage of relative activity. (*C*) Sustainment of the fully activated human ERR-γ by bisphenol E and bisphenol AF together with inverse agonist activity by 4-OHT. (*D*) Reversing activity of BPA, 4-α-cumylphenol, and 4-*tert*-butylphenol; the inverse agonist activity of 4-OHT was clearly reversed by all bisphenols tested in a dose-dependent manner. Data are from a single experiment performed in triplicate; two additional experiments gave similar results. High basal constitutive activity of ERR-γ was evaluated with the luciferase-reporter plasmid (pGL3/3 × ERRE), and the highest activity was estimated in a cell preparation of 1.0×10^5 HeLa cells/well.

Table 3. Receptor binding affinity (mean ± SE; $n = 3$) of BPA and its analogs for ER-α and their receptor selectivity for ERR-γ over ER-α.

Chemical	Binding affinity for ER-α (IC_{50}, nM)	ERR-γ receptor selectivity ER-α (IC_{50}, nM)/ERR-γ (IC_{50}, nM)
E_2	0.88 ± 0.13	Exclusively ER-α
Group A (chemicals as active as BPA for ERR-γ)		
Bisphenol E	ND	Exclusively ERR-γ
BPA	1,030 ± 146	105
4-α-Cumylphenol	4,770 ± 510	450
Group B (chemicals considerably potent for ERR-γ)		
Bisphenol B	246 ±29.7	9.46
4-*tert*-Butylphenol	ND	Exclusively ERR-γ
4-*tert*-Amylphenol	ND	Exclusively ERR-γ
4-Isopropylphenol	ND	Exclusively ERR-γ
Group C (chemicals moderately potent for ERR-γ)		
Bisphenol AP	361 ± 22.6	2.93
Bisphenol F	ND	Exclusively ERR-γ
4-*tert*-Octylphenol	925 ± 83.9	3.89
4-Ethylphenol	ND	Exclusively ERR-γ
Bisphenol AF	53.4 ± 7.28	0.15
Group D (chemicals extremely weak or inactive for ERR-γ)		
2,2-Diphenylpropane	ND	Inactive for both receptors
p-Cresol	ND	Almost inactive for both receptors
Phenol	ND	Inactive for both receptors

ND, not determined (IC_{50} value could not be calculated because of extremely weak binding activity even at a 10-μM concentration).

4-*tert*-butylphenol, 4-*tert*-amylphenol, and 4-isopropylphenol, and they were fully selective and specific for ERR-γ. In contrast, bisphenol B was very weakly active (246 nM) for ER-α, although it was still selective (about 9.5 times) for ERR-γ.

Among group C chemicals ($IC_{50}^{ERR\text{-}\gamma}$ = 120–350 nM), bisphenol F was almost completely inactive for ER-α, making it fully selective for ERR-γ (Table 3). This was also true for 4-ethylphenol. Bisphenol AP showed a weak binding affinity (361 nM) for ER-α, but it was still selective (about 3 times) for ERR-γ. However, bisphenol AF emerged as a ligand selective for ER-α with a selectivity ratio of 0.15 (Table 3). The reciprocal of 0.15 [i.e., ERR-γ (IC_{50})/ER-α (IC_{50}) = 6.67] denotes a selectivity ratio of bisphenol AF for ER-α.

The results clearly indicate that the alkyl groups on the central sp^3-C atom of bisphenol derivatives play a key role in selection of the NR ERR-γ and ER-α. When we checked the receptor binding activities of one series of bisphenol derivatives (i.e., bisphenol E, BPA, bisphenol B, bisphenol AP, and bisphenol AF), we found this line-up to be the order of compounds with increasing affinity to ER-α. At the same time, it was the order of compounds with decreasing affinity to ERR-γ. ERR-γ prefers the less bulky and less electrophilic alkyl groups, whereas ER-α appears to prefer the bulkier and more electrophilic alkyl groups.

4-*tert*-Octylphenol is a well-known endocrine disruptor candidate, but it was only moderately potent for ERR-γ (IC_{50} = 238 nM; Table 2). However, it was considerably weak for ER-α, with an IC_{50} of 925 nM; thus, we judged 4-*tert*-octylphenol to be somewhat selective (approximately 4 times) for ERR-γ. Another representative endocrine disruptor candidate is 4-nonylphenol, which was moderately active for ERR-γ (Takayanagi et al. 2006). Thus, 4-nonylphenol was slightly more selective for ERR-γ. However, some 4-alkyl phenols are distinctly more potent for ERR-γ than 4-*tert*-octylphenol and 4-nonylphenol: 4-*tert*-butylphenol, 4-*tert*-amylphenol, and 4-isopropylphenol. These 4-alkyl phenols are definitely novel candidates of the endocrine disruptor specific for ERR-γ.

Conclusion

In the present study we have shown that all the structural elements of BPA—the phenol and methyl groups and the phenyl group on the central sp^3-C atom—are prerequisite for binding to the NR ERR-γ. Furthermore, we have shown that the phenol derivatives are potent candidates for the endocrine disruptor that binds to ERR-γ. The binding affinity of [^{3}H]BPA to ERR-γ–LBD is extremely high, with a K_D value of 5.50 nM. Thus, it appears to be important to evaluate whether the previously reported effects of BPA at low doses are mediated through ERR-γ and its specific target gene(s).

At the same time, it is necessary to clarify the physiologic roles of ERR-γ and to examine the degree and ways in which BPA may influence these. This is particularly important because ERR-γ is expressed in a tissue-restricted manner—for example, it is expressed very strongly in the mammalian fetal brain and placenta—at sites that could have important outcomes for newborns. Recently, many lines of evidence have indicated that low doses of BPA affects the central nervous system (reviewed by vom Saal and Welshons 2005; Welshons et al. 2003, 2006). The molecular mechanism for these effects could involve, at least in part, the high affinity binding of BPA to ERR-γ. A similar phenomenon may be observed for other NRs, and the exploration of such chemical–receptor interactions requires a specific assay system or concept applicable to all the NRs.

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Designed modification of partial agonist of ORL1 nociceptin receptor for conversion into highly potent antagonist

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Abstract—Nociceptin is an endogenous agonist ligand of the ORL1 (opioid receptor-like 1) receptor, and its antagonist is a potential target of therapeutics for analgesic and antineuropathy drugs. Ac-RYYRIK-NH_2 is a hexapeptide isolated from the peptide library as an antagonist that inhibits the nociceptin activities mediated through ORL1. However, the structural elements required for this antagonist activity are still indeterminate. In the present study, we evaluated the importance of the acetyl-methyl group in receptor binding and activation, examining the peptides acyl-RYYRIK-NH_2, where acyl (R-CO) possesses a series of alkyl groups, R = C_nH_{2n+1} (n = 0–5). The isovaleryl derivative with the C_4H_9 (=$(CH_3)_2CHCH_2$–) group was found to reveal a high receptor-binding affinity and a strong antagonist nature. This peptide achieved a primary goal of eliminating the agonist activity of Ac-RYYRIK-NH_2 and producing pure antagonist activity.

1. Introduction

Nociceptin,[1] also known as orphanin FQ,[2] is a 17-mer neuropeptide with the sequence FGGFTGARKSARKLANQ. Nociceptin is an endogenous ligand of the ORL1 (opioid receptor-like 1) receptor, the structure of which is very similar to those of the δ, μ, and κ opioid receptors.[3] This receptor belongs to the G protein coupled receptor (GPCR) superfamily and couples specifically with G_i or G_o protein. Nociceptin induces hyperalgesia, and the nociceptin/ORL1 ligand–receptor system is also involved in many other physiological functions such as analgesia in the spinal cord and antiopioid effects in the brain.[1,2,4–6] The actions of nociceptin in the central nervous system also include the inhibition of locomotor activity and impairment of spatial learning.[7–9]

In general, for better understanding of such different functions of biologically active peptides, it is imperative to obtain a highly selective and specific receptor antagonist. Antagonist is an important and indispensable molecular tool for investigation of the inhibition mechanism of receptor activation. Because of the intrinsic hyperalgesic activity of nociceptin, its antagonists are expected to be highly effective analgesics.

Several different types of compounds have recently been identified as antagonists of nociceptin.[10] As full antagonists, a number of nonpeptide compounds have been designed and synthesized,[11] but it has been difficult to identify any general structural elements common to all of these organic compounds. As for compounds based on the structure of nociceptin peptide, [Phe[1]Ψ(CH_2–NH)Gly[2]]nociceptin(1–13)–NH_2[12] and [Nphe[1]]nociceptin-(1–13)–NH_2[13] have been reported as antagonists in the peripheral nervous system.[14–18] However, these

Abbreviations: Boc, *tert*-butyloxycarbonyl; Bpa, *p*-benzoyl-L-phenylalanine; BSA, bovine serum albumin; DMF, *N*,*N*-dimethylformamide; GPCR, G protein coupled receptor; HBTU, 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HOBt, 1-hydroxybenzotriazole; MALDI–TOF, matrix-assisted laser desorption ionization time-of-flight; MBHA, *p*-methylbenzhydrylamine; Nphe, *N*-benzylglycine; RP-HPLC, reversed-phase high performance liquid chromatography; RT, retention time; TFA, trifluoroacetic acid; Tris, tris(hydroxymethyl)aminomethane.

Keywords: Acyl group; Antagonist; Nociceptin; Structure–activity relationships.

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peptides appear to act as partial or even full agonists of nociceptin at the central nervous site.[19–22] UFP-101, the compound with the Leu^{14} → Arg and Ala^{15} → Lys substitutions in [Nphe^{1}]nociceptin-(1–13)–NH_2, is a competitive type of nociceptin antagonist.[23] This simultaneous Leu-Ala^{14,15} → Arg-Lys substitution was reported originally by us as a structural conversion to turn nociceptin into a super agonist.[24]

In addition to these nociceptin analogues, there is another type of antagonist compound selected from the peptide libraries. For instance, acetyl-hexapeptide amide Ac-Arg-Tyr-Tyr-Arg-Ile-Lys-NH_2 (Ac-RYYRIK-NH_2) has been reported as an effective nociceptin antagonist.[25] Since Ac-RYYRIK-NH_2 displaces [^{3}H]nociceptin in a dose-dependent manner, these two peptides should share and thus compete for the binding site in ORL1 receptor. However, Ac-RYYRIK-NH_2 per se was found to exhibit partial agonist activity in the [^{35}S]GTPγS binding assay.[26,27] In addition, it did not exhibit any in vivo activity, presumably due to the rapid degradation.

Our previous study of Ala-scanning for Ac-RYYRIK-NH_2 indicated that the N-terminal tripeptide Arg-Tyr-Tyr is crucially important for binding to the ORL1 receptor.[28] In the present study, based on the fact that the analogue lacking the acetyl group, H-RYYRIK-NH_2, shows drastically reduced binding efficacy (approximately 60-fold weaker than Ac-RYYRIK-NH_2), we noted the importance of the N-terminal acetyl group, CH_3CO–, as a structural element essential for binding to ORL1.[28]

The acetyl group has two different types of structural elements—the methyl (CH_3) group and the carbonyl (CO) group. In this study, focusing on the N-terminal acetyl-methyl group, we synthesized a series of acyl-RYYRIK-NH_2 peptides (acyl = R-CO, where the alkyl group is denoted as R = C_nH_{2n+1}; n = 0–5) (Table 1), and evaluated the structural effectiveness of the acyl–alkyl group (R) for the antagonist activity. We here describe the structure–activity relationships of acyl-RYYRIK-NH_2 peptides for the best selection of ORL1 nociceptin antagonism.

2. Results

2.1. Peptide syntheses

All of the 17 N-terminal modified hexapeptides, including the parent Ac-RYYRIK-NH_2, were synthesized by the manual solid-phase method using Fmoc-amino acids. Peptides in a pure form were obtained in an average yield of approximately 31%. Among the analogues, Ada-RYYRIK-NH_2 was obtained with the best yield of approximately 56%, while *t*-BuAc-RYYRIK-NH_2 was obtained with the worst yield of less than 10% (Table 1). These were all easily soluble in water and could be assayed without any trouble. Table 1 shows the analytical data of all analogues synthesized. The purity of the peptides was verified by analytical HPLC, in which all the peptides emerged with a single peak. The retention time (approximately 34 min) of the N-terminal modified analogues is much larger than that (23.24 min) of H-RYYRIK-NH_2. It is presumed that the increased hydrophobicity of acyl groups brings about an increased retention time on HPLC. The mass numbers measured were coincident with the values calculated (data not shown). Collectively, synthetic Ac-RYYRIK-NH_2 and its analogues have been found to reveal the authentic compounds.

2.2. ORL1 receptor fused with the G protein α_o subunit for better antagonism measurement

For efficient measurements of agonism and antagonism in the receptor responses, GPCR fused with the G pro-

Table 1. Synthetic yield and HPLC analytical data of acetyl-hexapeptide amide Ac-RYYRIK-NH_2, and its analogues

n	Structure of acyl = $C_nH_{2n+1}CO$ of Acyl-RYYRIK-NH_2	Name of acyl	Abbreviations of acyl	Yield (%)	RP-HPLC retention time (min)
0	HCO–	Formyl	For	34	24.89
1	CH_3CO–	Acetyl	Ac	52	26.16
2	CH_3CH_2CO–	Propionyl	Pr	28	28.33
3	$CH_3CH_2CH_2CO$–	Butyryl	Bu	20	29.88
	$(CH_3)_2CHCO$–	Isobutyryl	isoBu	39	29.85
4	$CH_3CH_2CH_2CH_2CO$–	Valeryl	Va	21	32.94
	$(CH_3)_2CHCH_2CO$–	Isovaleryl	isoVa	14	32.35
	$CH_3CH_2CH(CH_3)CO$–	2-Methylbutanoyl	MeBut	41	31.86
	$(CH_3)_3CCO$–	Pivaloyl	Piv	23	33.25
5	$CH_3CH_2CH_2CH_2CH_2CO$–	Hexanoyl	Hex	18	35.57
	$CH_3CH_2CH_2CH(CH_3)CO$–	2-Methylpentanoyl	2-MePen	29	35.15
	$(CH_3)_3CCH_2CO$–	*tert*-Butylacetyl	*t*-BuAc	8	34.73
	$CH_3CH_2C(CH_3)_2CO$–	2,2-Dimethylbutanoyl	2,2-diMeBut	37	34.21
	$(CH_3CH_2)_2CHCO$–	2-Ethylbutanoyl	EtBut	35	34.83
	C_6H_5CO–	Benzoyl	Bz	14	36.52
	$C_{10}H_{15}CO$–	Adamantyl	Ada	56	41.89

Three of the acyl groups are not listed, since those acyl chlorides or acids are not commercially available, and thus the peptides having those acyl groups were not chemically synthesized. These include the 3-methylpentanoyl ($CH_3CH_2CH(CH_3)CH_2CO$–), 4-methylpentanoyl ($(CH_3)_2CHCH_2CH_2CO$–), and 3,3-dimethylbutanoyl ($CH_3C(CH_3)_2CH_2CO$–) groups. Elution conditions for the analytical RP-HPLC to measure the retention time: solvent system, 0.1% aqueous TFA-(A solution) and acetonitrile containing 20% A solution-(B solution) with a gradient elution from 10% to 50% B solution for 40 min; flow rate, 0.5 ml/min; temperature, 25 °C; and UV detection, 230 nm.

tein α subunit has been recognized to afford an excellent assay system.[29] In the present study, we intended to establish such a system for the ORL1 receptor. Using human ORL1 receptor, we succeeded in preparing hORL1 fused with the G protein α_o subunit (hORL1-Gα_o) for both the receptor-binding assay and the functional in vitro biological assay. We first tested this assay system for nociceptin and Ac-RYYRIK-NH_2.

For the receptor-binding assay, the highest expression efficiency of hORL1-Gα_o receptor was pursued by using COS-7 cells for the ordinary ligand-saturation experiment. Under the best conditions using the tritium-labeled ligand [^{3}H]nociceptin, the largest specific binding was obtained by subtracting its nonspecific binding from the total binding. The data were analyzed by Scatchard plot analysis, and the dissociation constant K_d was calculated to be 0.37 nM, being almost the same as the K_d value (0.41 nM) reported previously for solo rat ORL1 receptor with no G protein fused.[28] Also, in this study almost the same result (K_d = 0.40 nM) was obtained for human ORL1 receptor with no G protein fused. The results imply that both G protein fused and nonfused receptors interact with [^{3}H]nociceptin equally well.

In the ligand–receptor competitive binding assay, Ac-RYYRIK-NH_2 exhibited a very high affinity, with an IC_{50} value of 0.79 nM (Table 2). This result indicates that Ac-RYYRIK-NH_2 binds to the ORL1 receptor very strongly, and that its binding ability is almost equivalent to that of nociceptin itself (IC_{50} = 0.60 nM).

The in vitro functional activity was evaluated by measuring the fold-stimulation of [^{35}S]GTPγS binding. The activity was compared with that of nociceptin (EC_{50} = 3.91 nM) (Table 2). The extent of [^{35}S]GTPγS binding in the presence of nociceptin was at least 10 times greater than that in its absence. When the [^{35}S]GTPγS binding of the parent acetyl containing hexapeptide Ac-RYYRIK-NH_2 was measured, it was estimated to reveal approximately 60% stimulation of the maximum response by nociceptin. Obviously, Ac-RYYRIK-NH_2 is a partial agonist, and yet it possesses considerably strong agonist activity. The EC_{50} value of Ac-RYYRIK-NH_2 was estimated to be 12.9 nM, the activity of which is only approximately threefold weaker than nociceptin.

Table 2. Binding potency and biological activity of nociceptin, acetyl-hexapeptide amide Ac-RYYRIK-NH_2, and its analogues for the human ORL1 receptor fused with Gα protein

Peptides acyl-RYYRIK-NH_2 (acyl groups)	ORL1 receptor 1 binding potency IC_{50} (nM)	[^{35}S]GTPγS binding activity	
		EC_{50} (nM)	E_{max} (%)
Nociceptin	0.60 ± 0.08	3.91 ± 0.34	100
H-	218 ± 78	N.D.	—
For-	0.66 ± 0.09	23.3 ± 4.2	61 ± 2.3
Ac-	0.79 ± 0.18	12.9 ± 2.8	58 ± 3.2
Pr-	1.70 ± 0.66	27.6 ± 6.8	46 ± 3.6
Bu-	1.86 ± 0.60	32.4 ± 12.1	21 ± 3.0
isoBu-	2.81 ± 0.52	44.5 ± 11.7	14 ± 3.8
Va-	5.67 ± 0.26	N.D.	7 ± 2.1
isoVa-	7.42 ± 0.87	N.D.	≈0
MeBut-	21.3 ± 2.3	52.7 ± 16.8	27 ± 1.8
Piv-	14.9 ± 3.5	97.9 ± 20.5	32 ± 5.6
Hex-	21.7 ± 5.3	N.D.	9 ± 2.6
2-MePen-	47.3 ± 8.0	23.8 ± 4.7	17 ± 4.7
t-BuAc-	16.9 ± 4.1	46.5 ± 2.0	33 ± 2.0
2,2-diMeBut-	45.5 ± 7.1	17.7 ± 3.4	17 ± 3.4
EtBut-	92.6 ± 5.4	N.D.	10 ± 1.5
Bz-	14.7 ± 2.6	18.3 ± 4.6	38 ± 5.8
Ada-	3.42 ± 0.49	19.2 ± 5.9	35 ± 4.9

For the receptor-binding assay, [^{3}H]nociceptin was used as a tracer. Data are means ± SEM of at least three experiments (n = 3–8). N.D. (not determined) means that the activity (EC_{50} (nM)) was not calculated due to inactivity.

2.3. Activities of acyl-RYYRIK-NH_2 peptides with non-branched acyl–alkyl groups

In the binding assay using rat ORL1 receptor, the N-terminal free analogue of Ac-RYYRIK-NH_2, namely, H-RYYRIK-NH_2, exhibited a drastically diminished binding potency.[28] A similar result was obtained from the assay using the G protein-fused receptor hORL1-Gα_o. H-RYYRIK-NH_2 showed an IC_{50} value of 218 nM, indicating that it is approximately 280-fold less active than Ac-RYYRIK-NH_2 (Table 2). Since these results clearly indicate that ORL1 receptor possesses a specific binding site for the acetyl group (CH_3CO) of Ac-RYYRIK-NH_2, we attempted to optimize the acyl–alkyl group. We designed and synthesized a series of analogues, in which the acetyl group was substituted with the acyl groups (R-CO) of different alkyl groups (R = C_nH_{2n+1}; n = 0–5): i.e., For (n = 0, R = H), Pr (2, CH_3CH_2), Bu (3, $CH_3CH_2CH_2$), Va (4, $CH_3CH_2CH_2CH_2$), and Hex (5, $CH_3CH_2CH_2CH_2CH_2$) (Table 1).

In the ligand–receptor-binding assay, For-RYYRIK-NH_2 exhibited the highest affinity (IC_{50} = 0.66 nM) (Table 2) among all the analogues, including Ac-RYYRIK-NH_2. Since For-RYYRIK-NH_2 is almost equipotent with nociceptin (IC_{50} = 0.60 nM) in displacing [^{3}H]nociceptin, their total binding energies to attach to ORL1 must be similar to each other. As shown in Figure 1, they exhibited almost the same dose–response curves, revealing a similar binding mode. Other analogues exhibited rather weaker binding affinity as compared with the parent peptide Ac-RYYRIK-NH_2 (Table 2).

Figure 2 shows the activity profiles of nociceptin, Ac-RYYRIK-NH_2, and its analogues in the [^{35}S]GTPγS binding assay. By using the membrane preparations from the cells expressing hORL1-Gα fusion receptor, nociceptin exhibited a strong binding activity, indicating that nociceptin stimulates the G-protein activation in a dose-dependent manner. Ac-RYYRIK-NH_2 exhibited considerably high activity, and its maximum response reached 60% of that by nociceptin. For-RYYRIK-NH_2 elicited almost the same response as Ac-RYYRIK-NH_2. In contrast to their agonist activity, the maximum response of Pr-RYYRIK-NH_2 and Bu-RYYRIK-NH_2 was significantly reduced by 20% and 40%, respectively. Furthermore, it was found that Va-RYYRIK-NH_2 and Hex-RYYRIK-NH_2 were virtually devoid of agonist activity (Fig. 2). Apparently, the receptor efficacy

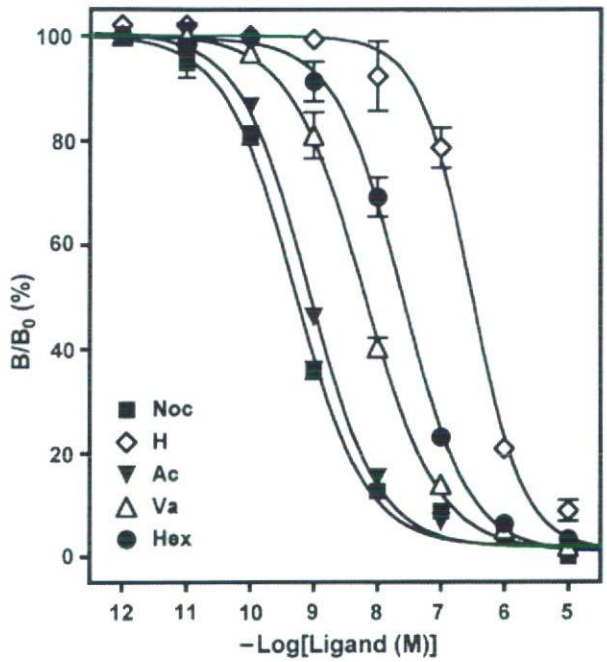

Figure 1. Dose–response curves of nociceptin and acyl-RYYRIK-NH_2 peptides in the binding assay for the hORL1-Gα fusion protein. The receptor tracer is [^{3}H]nociceptin (0.05 nM in the final concentration). The curves are of nociceptin and the parent Ac-RYYRIK-NH_2 and the analogues of acyl-RYYRIK-NH_2 with the N-terminal acyl–alkyl group R = C_nH_{2n+1}: H- (n = 0), Va- (n = 4), and Hex- (n = 5).

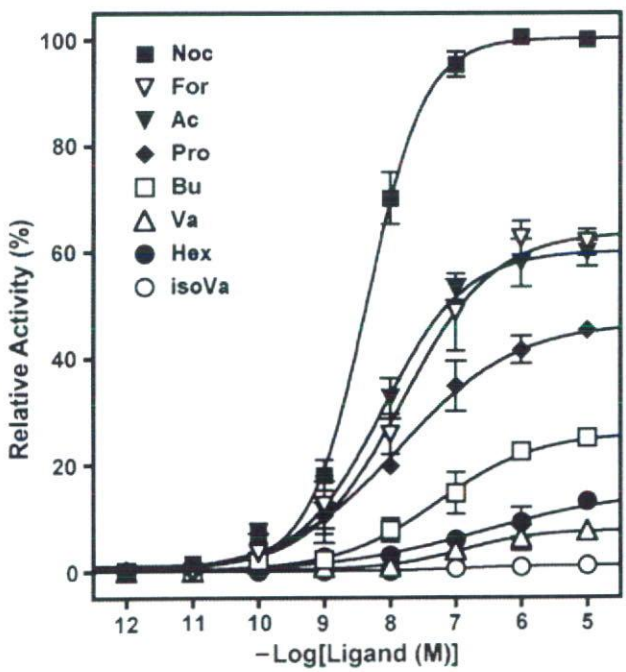

Figure 2. Dose–response curves of nociceptin and acyl-RYYRIK-NH_2 peptides in the [^{35}S]GTPγS binding assay using the hORL1-Gα receptor. Assayed acyl-RYYRIK-NH_2 peptides are with the acyl–alkyl group R = C_nH_{2n+1} (n = 0–5). Data are means ± SEM of at least five experiments.

decreased as the chain length increased. Here, it should be noted that Hex-RYYRIK-NH_2 having a hexanoyl ($CH_3CH_2CH_2CH_2CH_2CO$ = $C_5H_{11}CO$) group is clearly stronger than Va-RYYRIK-NH_2 having a valeryl ($CH_3CH_2CH_2CH_2CO$ = C_4H_9CO) group.

2.4. Activities of acyl-RYYRIK-NH_2 peptides with branched acyl–alkyl groups

In the [^{35}S]GTPγS binding assay (valeryl=)$CH_3CH_2CH_2CH_2CO$-RYYRIK-NH_2 was most active among acyl-RYYRIK-NH_2 peptides. We next examined a series of acyl-RYYRIK-NH_2, which have the acyl group (C_nH_{2n+1}-CO, n = 3–5) with eight different branched alkyl groups. Those include the acyl groups such as isobutyryl (isoBu, $(CH_3)_2CHCO$–), isovaleryl (isoVa, $(CH_3)_2CHCH_2CO$–), 2-methylbutanoyl (MeBut, $CH_3CHCH(CH_3)CO$–), pivaloyl (Piv, $(CH_3)_3CCO$–), 2-methylpentanoyl (2-MePen, $CH_3CH_2CH_2CH(CH_3)CO$–), *tert*-butylacetyl (*t*-BuAc, $(CH_3)_3CCH_2CO$–), 2,2-dimethylbutanoyl (2,2-diMeBut, $CH_3CH_2C(CH_3)_2CO$–), and 2-ethylbutanoyl (EtBut, $(CH_3CH_2)_2CHCO$–) (Table 1). The derivatives with the 3-methylpentanoyl ($CH_3CH_2CH(CH_3)CH_2CO$–) and 4-methylpentanoyl ($(CH_3)_2CHCH_2CH_2CO$–) groups were not prepared because their chlorides or acids were not commercially available. In contrast, a totally different type of acyl group, benzoyl (Bz, C_6H_5CO–) and adamantyl (Ada, $C_{10}H_{15}CO$–), was selected to assess the unique structural properties that contribute to receptor binding and activation.

When the binding ability of these acyl-substituted analogues was tested, it was found that the molecular size of the acyl–alkyl groups greatly affects the receptor-binding affinity (Table 2). The EtBut group induced the weakest activity, showing an approximately 120-fold decrease in binding affinity as compared with the parent compound Ac-RYYRIK-NH_2. Analogues having larger alkyl groups exhibited a weaker binding potency. It seems that the binding site for acyl–alkyl is not so large as to bind bulkier groups such as EtBut, 2,2-diMeBut, and 2-MePen.

Unexpectedly, the *tert*-butylacetyl (*t*-BuAc) derivative showed an affinity stronger than the compounds having acyl groups with the same molecular weight. This difference must be due to the very compact structure of the *t*-BuAc group. It was also found that Ada-RYYRIK-NH_2 is very potent (IC_{50} = 3.42 nM), despite the presence of the large acyl–alkyl $C_{10}H_{15}$. This unexpectedly high binding potency of Ada-RYYRIK-NH_2 must be due to the compactness of the adamantyl group. The π–electron rich benzoyl-protected compound Bz-RYYRIK-NH_2 also exhibits considerably strong binding potency (14.7 nM, Table 2). Collectively, the binding site for the acyl–alkyl group in the ORL-1 receptor appears to be the size of three carbons in the acyl-backone (4–5 Å) with several methyl groups.

In the [^{35}S]GTPγS binding assay, these analogues having bulky acyl groups still exhibited moderate receptor activation activity (EC_{50} = 20–50 nM and E_{max} = 10–40%, Table 2). Also, Bz-RYYRIK-NH_2 and Ada-RYYRIK-NH_2 showed an approximately 40% stimulatory response with EC_{50} of approximately 20 nM (Table 2). These results suggest that there is a space to capture

the bulky alkyl groups present at the N-terminus of R-CO-RYYRIK-NH_2, but their interaction with the ORL1 receptor is very subtle and vague, either stimulating or blocking the receptor activation.

To obstruct the receptor activation, the best-fitting alkyl group appears to be the isovaleryl (denoted as isoVa) group. The peptide isoVa-RYYRIK-NH_2 showed almost a negligible response at its 0.10–10 μM concentrations. Its E_{max} value at the 10 μM concentration was less than 5%, and thus it was impossible to estimate the EC_{50} value. It should be noted that branched isoVa-RYYRIK-NH_2 is clearly less potent than non-branched Va-RYYRIK-NH_2 in this [^{35}S]GTPγS binding assay.

2.5. Antagonist activity estimated by [^{35}S]GTPγS binding to human ORL1-Gα receptors

Because of a moderately high receptor-binding affinity (IC_{50} = 7.42 nM) and a low receptor activation efficacy, isoVa-RYYRIK-NH_2 appeared to be an efficient antagonist against nociceptin/ORL1. We then examined its competitive antagonism in the [^{35}S]GTPγS binding assay. For the Schild analysis, nociception was assayed in the presence of isoVa-RYYRIK-NH_2. Three series of dilutions of nociceptin were tested with different concentrations (10^{-8}, 10^{-7}, and 10^{-6} M concentrations, respectively) of isoVa-RYYRIK-NH_2. The assay solution was incubated for 60 min, with the expectation that competition would occur between nociceptin and isoVa-RYYRIK-NH_2 to reside in the ORL1 receptor.

As shown in Figure 3A, solo nociceptin demonstrates a superlative dose-dependent sigmoid curve. This nociceptin's concentration–response curve shifted rightward in the presence of isoVa-RYYRIK-NH_2, indicating that isoVa-RYYRIK-NH_2 occupies some population of ORL1 receptor. As the concentrations of isoVa-RYYRIK-NH_2 increased, the occupied population increased, resulting in a further rightward shift of the nociceptin curve (Fig. 3A). Eventually, the Schild analysis determined isoVa-RYYRIK-NH_2 as a potent competitive antagonist, with the pA_2 value calculated to be 8.80 (Fig. 3B). This analogue is three orders of magnitude more potent as an antagonist than [Nphe^1]nociceptin(1–13)–NH_2, and as potent as the nonpeptide J-113397,[30] the most potent antagonist reported to date.

We also examined the competitive antagonism of Va-RYYRIK-NH_2 in the [^{35}S]GTPγS binding assay. This compound shifted the nociceptin curve similar to that of isoVa-RYYRIK-NH_2, and its pA_2 value was calculated to be 8.51. Thus, together with the fact that Va-RYYRIK-NH_2 retains a weak agonist activity in the [^{35}S]GTPγS binding assay in spite of almost the complete inactivity of isoVa-RYYRIK-NH_2, we concluded that isoVa-RYYRIK-NH_2 is slightly, but definitely, more potent as an antagonist than Va-RYYRIK-NH_2 in the [^{35}S]GTPγS binding assay. As a result, the present results indicate that isoVa-RYYRIK-NH_2 is the best antagonist among the N-terminal modified hexapeptides.

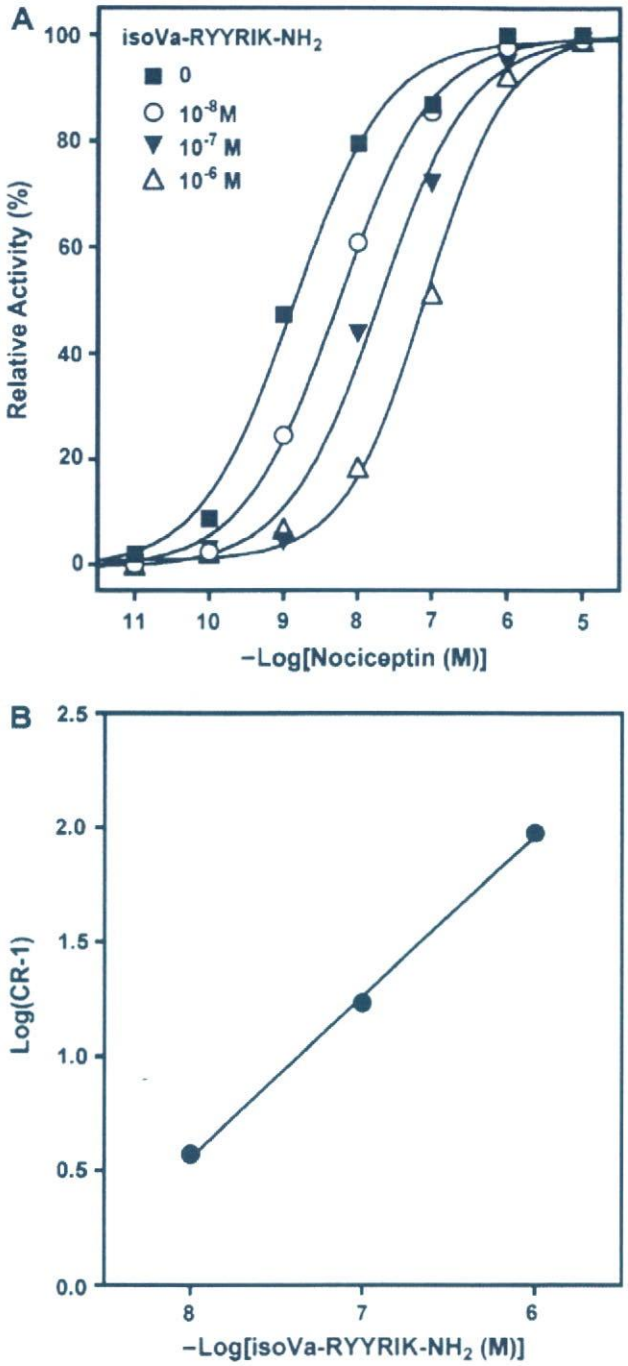

Figure 3. The antagonist activity isoVa-RYYRIK-NH_2 in the [^{35}S]GTPγS binding assay (A) and the Schild plot analysis of this assay (B). The extrapolated pA_2 value from the plot analysis was calculated to be 8.80 ± 0.20.

2.6. Antagonist activities of analogues in the mouse vas deferens

Nociceptin exerts inhibitory effects in electrically stimulated preparations such as the guinea pig ileum (GPI) and MVD.[31,32] In the present study, we established an assay system in which nociceptin inhibits the electrically evoked contractions of the MVD in a concentration-dependent manner. Its maximal effect was an approximately 80% reduction of the control contraction, and

the ED_{50} value was estimated to be 19.3 nM, almost the same as the reported value.[31] This agonist activity of nociceptin appears to be due to the specific interaction with ORL1 receptor in MVD, since nociceptin exhibited absolutely no binding affinity for the opioid receptors.

In contrast to the considerably high agonist activity in the GTPγS binding assay, Ac-RYYRIK-NH_2 was almost completely inactive in MVD. It exhibited only weak agonist activity (an approximately 40% reduction, but only at its highest concentration (10 μM)). Amino-free derivative H-RYYRIK-NH_2 did not at all modify the electrically induced twitches of MVD. Most of the other analogues were also found to be almost completely inactive. By contrast, Pr-RYYRIK-NH_2 and Bz-RYYRIK-NH_2 exhibited considerably high activity (over 90% inhibition of the electrically evoked twitches) all at once at their 10 μM concentration. This abrupt activity was eventually assumed to be due to their binding to the δ opioid receptor (data not shown).

The antagonist ability of acyl-RYYRIK-NH_2 analogues in the MVD assay was assessed by co-administration with nociceptin at specific concentrations. It was found that isoVa-RYYRIK-NH_2 shifts the dose–response curve of nociceptin rightward in a concentration-dependent manner, the curves being parallel to the control (Fig. 4A). This shift demonstrates that isoVa-RYYRIK-NH_2 occupies the binding site of the receptor to which nociceptin competitively binds. The extrapolated pA_2 value from the Schild plot analysis was calculated to be 9.70 (Fig. 4B). Together with the prominent antagonist activity in the GTPγS binding assay, strong antagonist activity in the MVD assay suggests that isoVa-RYYRIK-NH_2 is a highly potent competitive antagonist for nociceptin.

3. Discussion

3.1. Assay system to evaluate the antagonist activity of Ac-RYYRIK-NH_2 analogues

In this study, we attempted to develop a potent peptidic antagonist of ORL1 receptor. For evaluation of antagonist activities, there are two different types of in vitro assays—the GTPγS binding assay and the MVD muscle assay. Since the GTPγS binding assay is carried out for the recombinant receptor preparations, especially by using G protein-fused receptor (hORL1-$G\alpha_o$ in this study), we are able to evaluate the ability of the compound solely for a single type of receptor. By contrast, the muscle preparations usually contain several different types of receptor families. For instance, MVD consists of all three opioid receptor subtypes, particularly the δ-opioid receptor,[31] in addition to the ORL1 receptor. In the present study, in both the GTPγS binding assay and the MVD muscle assay, isoVa-RYYRIK-NH_2 was almost completely inactive and at the same time showed specific antagonist activity, indicating that this compound is a genuine antagonist of the nociceptin/ORL1 ligand–receptor system. This result is in sharp contrast to the activities of the parent compound Ac-RYYRIK-NH_2, which is partially active in both the assays.

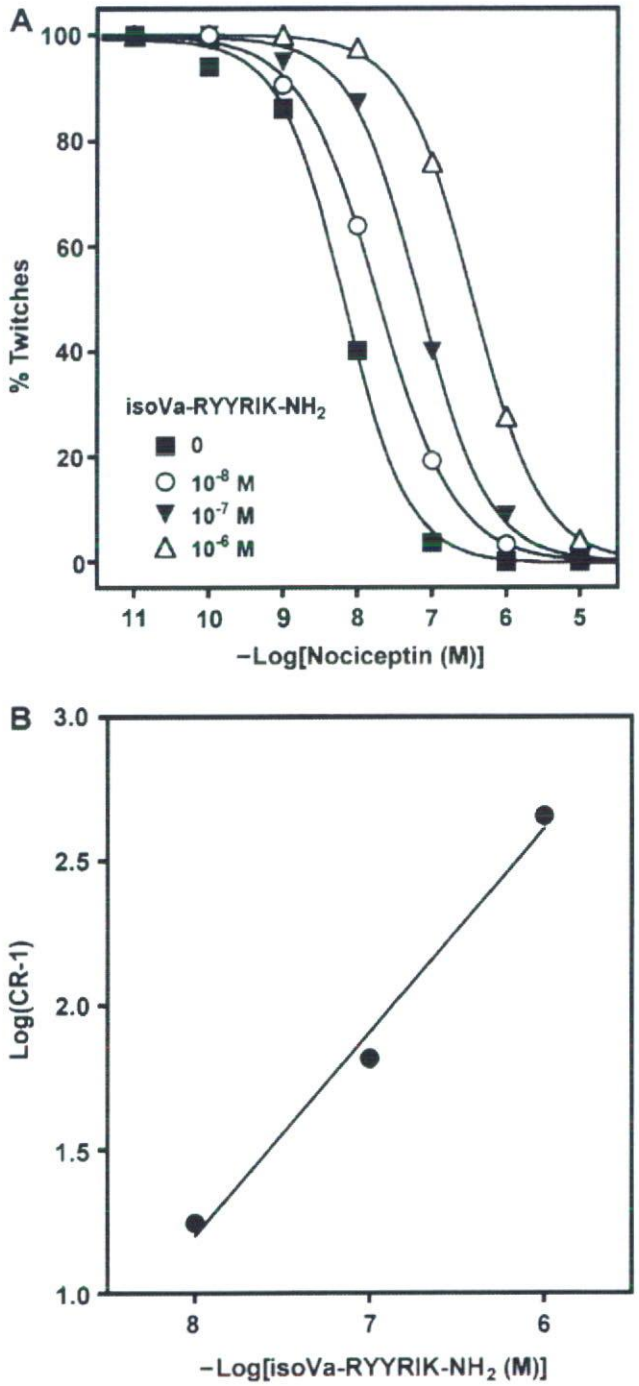

Figure 4. The antagonist activity isoVa-RYYRIK-NH_2 in the MVD muscle assay (A) and the Schild plot analysis of this assay (B). The extrapolated pA_2 value from the plot analysis was calculated to be 9.70 ± 0.32.

Antagonist with no agonist activity is a highly specific molecular tool important for exploration of the inhibition mechanism of receptor activation. We first tried to establish the proper assay system to evaluate the antagonist activity. The results described above indicate that the assays using GTPγS-fused receptor are fundamentally important for evaluating both agonist and

antagonist activities. Since the report indicated that there are some species differences between human and rodent ORL1 receptors,[10] we examined both types. However, in the present study we found no crucial differences in the receptor binding and the GTPγS binding assays using rat and human ORL1 receptors. As long as we use recombinant ORL1 receptors, it appears to be possible to use the receptor molecules of any aminal species.

Eventually we decided to utilize the human ORL1 receptor to evaluate the activities of acyl-RYYRIK-NH_2 series. In order to more effectively assess G-protein activation, we fused ORL1 with the G protein α subunit. Obviously, fusion genes between the GPCR receptor molecule and its coupled G protein α-subunit do not exist in nature. However, such fusion proteins show a much enhanced signaling efficiency in the cells transfected. Indeed, the GTPγS-fused ORL1 receptor exhibited a high stimulatory enhancement in a dose-dependent manner much more effectively than the ORL1 receptor with no GTPγS fusion. The present results are the first data obtained from assays using the human GTPγS-fused ORL1 receptor, the assay using a rat GTPγS-fused ORL1 receptor being reported by Molinari et al.[33] This assay system enabled us to estimate the detailed activation levels against the acyl-RYYRIK-NH_2 series having similar N-terminal acyl groups.

3.2. Structural determinants of N-terminal acyl–alkyl group for antagonist activity

We attempted to optimize the size of the N-terminal acyl group that may fit the binding pocket of the human ORL1 receptor. We first selected a linear acyl–alkyl group series for acyl-RYYRWK-NH_2. As shown in Table 2, the highest receptor-binding activity was attained by For-RYYRIK-NH_2 (IC_{50} = 0.66 nM), but it immediately became apparent that even Ada-RYYRIK-NH_2 shows significantly potent binding ability (3.42 nM) to the ORL1 receptor. Since the N-terminal free analogue H-RYYRIK-NH_2 is intrinsically inactive, these results imply that one of the most important structural elements for the acyl group in acyl-RYYRIK-NH_2 is the binding of the carbonyl group (C=O) to the ORL1 receptor. Since the different sizes of the acyl–alkyl group afford different strengths of antagonist activity, the size of the alkyl group appears to be the determinant of the inability of acyl-RYYRIK-NH_2 to activate the receptor as a basal condition of the antagonism. Our results indicate that there is an optimal size of alkyl group for the antagonism. The analogue having the vareryl group, Va-RYYRIK-NH_2, was found to be the strongest antagonist among a series of C_nH_{2n+1}-CO-RYYRIK-NH_2 with the non-branched alkyl (C_nH_{2n+1}) group.

Although the literal reason is not apparent, N-terminal modification has also been reported for Ac-RYYRWK-NH_2,[34,35] a derivative of Ac-RYYRIK-NH_2. The length of the aliphatic methylene chain was characterized as a determinant of efficacy, which decreases with acetyl through pentanoyl(=vareryl) and then increases up to heptanoyl. However, in the present study, isoVa-RYYRIK-NH_2 was eventually found to be the strongest antagonist. As an antagonist, isoVa-RYYRIK-NH_2 was definitely stronger in the GTPγS binding assay than Va-RYYRIK-NH_2. It is clear that the length of the aliphatic methylene chain is not merely a determinant of the receptor efficacy. The important determinant for the antagonism induction is the molecular size and shape of the acyl–alkyl group.

When the biological activities of Bu(=$CH_3CH_2CH_2CO$)-RYYRIK-NH_2 (E_{max} = 21%), isoVa(=$(CH_3)_2CHCH_2CO$)-RYYRIK-NH_2 (≈0%), and *t*-BuAc(=$(CH_3)_3CCH_2CO$)-RYYRIK-NH_2 (33%) were compared (Table 2), only isoVa-RYYRIK-NH_2 was found to be a pure antagonist. The methyl branching at the Cγ position is a crucial determinant for the antagonism, indicating that the binding site for the isovareryl group with the N-terminal $(CH_3)_2CHCH_2$– captures or arrests the peptide RYYRIK-NH_2 so as to not activate the receptor.

3.3. A possible binding site of isoVa-RYYRIK-NH_2

By the photo-affinity labeling method using [Bpa^2, ^{125}I-Tyr^3]Ac-RYYRWK-NH_2, the binding site of Ac-RYYRWK-NH_2 was suggested to be the portion limited from Gln^{107} in the transmembrane #2 (TM2) to Leu^{113} in the extracellular loop #1 (EL1) of human ORL1 receptor, Gln-Gly-Thr-Asp-Ile-Leu-Leu.[36] This portion is different from the nociceptin-binding site reported by Mouledous et al.[37] They utilized [Bpa^{10}, ^{125}I-Tyr^{14}]nociceptin for labeling and identified the portion of ORL1[296–302] (Thr^{296}-Ala-Val-Ala-Ile-Leu-Arg^{302}, EL3-TM7) as a nociceptin-binding site. There is no overlap between these two binding sites. However, these two ligands, Ac-RYYRI(or W)K-NH_2 and nociceptin, should share the same binding site, at least in part, because they can displace [^{3}H]nociceptin in the human ORL1 receptor-binding assay.

Displacement of [^{3}H]nociceptin is feasible only when acyl-RYYRIK-NH_2 or acyl-RYYRWK-NH_2 occupies the same receptor site for [^{3}H]nociceptin in ORL1. Thus, if the portions ORL1[107–113] and ORL1[296–302] are specific for acyl-RYYRI(or W)K-NH_2 and nociceptin, respectively, the binding site shared by these peptides must exist in region(s) other than these portions in ORL1. Both acyl-RYYRWK-NH_2 and nociceptin are rich in basic amino acids (Arg and Lys). The most likely portion shared by these peptides is to bind such residues rich in basic amino acids.

One of the most important residues in acyl-RYYRI(or W)K-NH_2 and nociceptin is their N-terminus. The N-terminal free amino group is essential for nociceptin to bind to ORL1 for the receptor activation, but the N-terminal acyl group, particularly the isovaleryl group, is crucial to bind to ORL1 for the receptor inactivation. To identify the particular receptor site for binding of the N-terminal region is key to revealing the receptor activation/inactivation mechanisms of ORL1 receptor.

isoVa-RYYRIK-NH_2 is definitely superior to the peptide and nonpeptide antagonists reported for ORL1 receptor to date. Among the peptide antagonists, [Nphe^1]nociceptin(1–13)–NH_2 has been announced as a pure and potent antagonist. However, as a pure antagonist, isoVa-RYYRIK-NH_2 is much more potent (three orders of magnitude more potent) than [Nphe^1]nociceptin(1–13)–NH_2. When compared with the most potent antagonist of J-113397,[30] isoVa-RYYRIK-NH_2 was found to be as active as this nonpeptide J-113397, exhibiting almost the same antagonist activity. The ligand-binding site of ORL1 receptor is thought to be different between the peptide ligand and the nonpeptide ligand. Thus, the usefulness of isoVa-RYYRIK-NH_2 as an antagonist should be emphasized as a specific competitor of nociceptin. This is particularly important to elucidate the molecular mechanism of the nociceptin/ORL1 ligand–receptor system.

4. Conclusion

isoVa-RYYRIK-NH_2 was found to be an efficient nociceptin antagonist with high affinity for the ORL1 receptor. isoVa-RYYRIK-NH_2 is also significant due to its pure antagonist activity. These results were obtained by the two different efforts to eliminate the agonist activity of Ac-RYYRIK-NH_2 and to retain antagonist activity. isoVa-RYYRIK-NH_2 appears to be a valuable molecular tool in structure–activity studies for the nociceptin/ORL1 ligand–receptor system.

5. Experimental

5.1. Peptide syntheses

All peptides used in this study were synthesized (0.15 mmol scale) by the manual solid-phase method using Fmoc-chemistry. Peptides were synthesized using Fmoc-NH-SAL resin, and the coupling reaction was carried out with 2-(1*H*-benzotriazole)-1-1,3,3-tetramethyluronium hexafluorophosphate (HBTU) in the presence of 1-hydroxy benzotriazole (HOBt) dissolved in *N*-methylpyrrolidone (NMP) and *N*,*N*-dimethylformamide (DMF). Each coupling reaction was examined for completion by means of the Kaiser ninhydrin test. N-terminal modifications of acylation were carried out at the end of each synthesis cycle by using acyl chloride (R-CO–Cl). A N-terminal free analogue of Ac-RYYRIK-NH_2 was obtained together with the parent peptide without acetylation.

After completion of the synthesis, the peptides were liberated from the resin using a cocktail reagent containing 90% trifluoroacetic acid (TFA), 2.5% water, 5% phenol, 5% thioanisole, and 2.5% ethanedithiol. Crude peptide was purified by gel filtration on a column (2.0 × 100 cm) of Sephadex G-15 (Pharmacia Biotech, Uppsala, Sweden) eluted with 10% acetic acid. For further purification, reversed-phase high performance liquid chromatography (RP-HPLC) was carried out on a preparative HPLC column (25 × 250 mm; Cica-Merck LiChrospher RP-18 (e), 5 μm). The linear elution conditions employed were as follows: solvent system, 0.1% aqueous TFA-(A solution) and acetonitrile containing 20% A solution-(B solution); flow rate, 5 ml/min; temperature, 25 °C; and UV detection, 230 nm.

The peptide purity was verified by analytical RP-HPLC (4 × 250 mm, Cica-Merck LiChrospher 100 RP-18, 5 μm) using the same elution conditions, except for a flow rate of 0.5 ml/min. The mass spectra of peptides were measured on a mass spectrometer Voyager™ DE-PRO (PerSeptive Biosystems Inc., Framingham, MA, USA) using the matrix-assisted laser desorption ionization time-of-flight (MALDI–TOF) method.

5.2. Cell culture and transfection

All receptors were transfected in COS-7 cells with human receptor cDNA. The cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) in the 100 U/ml penicillin, and 100 μg/ml streptomycin. The plasmid DNAs (20 μg) of human ORL1-Gα receptor were transiently transfected into 90% confluent COS-7 cells (0.5×10^5 per cm^2) in a 60 cm^2 culture plate by using TransFectin Lipid Reagent (Bio-Rad Laboratories, Hercules, CA, USA). After 48 h, cells were harvested and centrifuged for 10 min at 500*g* (4 °C).

Cells were then resuspended in the buffer containing 5 mM Tris–HCl, 1 mM EGTA, 1 mM dithiothreitol (DTT), and 11% saccharose (pH 7.4), and homogenized with the Potter–Elvehjem homogenizer (50 strokes). The homogenate was centrifuged for 10 min at 1000*g* (4 °C). The supernatant was centrifuged again for 20 min at 24,000*g* (4 °C), and the pellet was washed with the buffer containing 5 mM Tris, 1 mM EGTA, and 1 mM DTT (pH 7.4). The concentration of membrane protein was estimated by the BCA protein assay method using bicinchoninic acid (Pierce, Rockford, IL, USA). The prepared membrane was frozen at −80 °C until use.

5.3. Receptor-binding assay

The receptor-binding assay with cell membranes was conducted in a 96-well format. The receptor-binding potencies of synthetic peptides were assessed by the radio-ligand receptor-binding assay using COS-7 cell membrane preparations expressing human ORL1-Gα fusion receptors. Each well of the 96-well plate (300 μl) containing 2–3 μg/ml membrane protein, a series of concentrations of synthetic peptide, and 0.05 nM [^{3}H]nociceptin (158 Ci/mmol; Perkin-Elmer Life and Analytical Sciences, Boston, MA) were incubated for 90 min at 25 °C in 50 mM Hepes–Tris buffer (pH 7.4) containing 0.1% bovine serum albumin (BSA). Bacitracin (100 μg/ml) was added as a protease inhibitor. To coat the filter surface, plates were soaked in 0.5% ethyleneimine polymer aqueous solution for 30 min.

After incubation, the mixture was filtered through the glass fiber UniFilter GF/B plate using the FilterMate Harvester (Packard Instrument, Meriden, CT, USA).

Twenty microliters of MicroScinti40 (Packard) was added to each well. The plates were sealed with TopSeal (Packard) and read on the TopCount (Packard) for 3 min per well. The computer program ALLFIT[38] was used to draw dose–response curves for the analysis. The binding potency of each peptide was estimated as the IC_{50} value, the peptide concentration at which the half-maximal inhibition is achieved.

5.4. [^{35}S]GTPγS binding assay

The in vitro biological activity of synthetic peptides was assessed by the [^{35}S]GTPγS binding assay. Receptor-mediated G-protein activation was measured as described previously.[28] The membranes (5–10 μg) were suspended in 50 mM Hepes–Tris buffer (pH 7.4) containing 100 mM NaCl, 10 mM $MgCl_2$, 200 μM EGTA, and 200 μM DTT. Each well (100 μl) was incubated for 1 h at 25 °C with peptides of appropriate concentrations in the presence of 3 μM GDP and 200 pM of [^{35}S]GTPγS (1000 Ci/mmol, GE Healthcare Biosciences, Buckinghamshire, UK). Nonspecific binding was determined in the presence of 10 μM GTPγS.

After incubation, the reaction mixture was filtered through the glass fiber UniFilter GF/B plate and washed in a similar manner as described for the radio-ligand receptor-binding assay. The activity was estimated by calculating the EC_{50} value, which exhibits the concentration inducing a 50% activity of its own maximal stimulation. The antagonist activity was measured by the concentration–response curves of the nociceptin, which were pictured in both the absence and presence of increasing concentrations of the test compound. The pA_2 value was also estimated to reveal antagonist activity according to the method of Arunlakshana and Schild.[39]

5.5. MVD muscle assays

The in vitro biological assay was carried out using mouse vas deferens (MVD) of male ICR mouse (25–35 g) as described by Hughes et al.[40] The tissue was mounted in a 5-ml organ bath (Panlab s.l., Barcelona, Spain) containing aerated (95% O_2/5% CO_2) Krebs–Ringer solution ((concentrations in mM) NaCl 118.5, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, $CaCl_2$ 1.8, and glucose 10) at 37 °C. An initial tension of 300 mg was applied. The tissue was stimulated between the alloy wire electrodes using pulses of 1-ms duration with a frequency of 0.1 Hz at the maximal voltage. The electrically induced contractions were recorded using a force transducer (Panlab s.l.) and a PowerLab/4sp (ADInstruments Pty, Chastle Hill, Australia) multichannel polygraph. Digital stimulators (Panlab s.l.) were used for the electrical stimulation.

The agonist potency of compounds was determined by depicting a concentration–response curve to calculate the ED_{50} value. The percent inhibition of the stimulation-induced contraction produced by each agonist was plotted against the log agonist concentration. ED_{50} is defined as the concentration of agonist producing 50% of the maximum effect attainable by that agonist. For experiments to measure the antagonism, the test sample was added to the bath 15 min prior to addition of nociceptin as agonist. The concentration–response curves of the agonist were pictured in both the absence and presence of increasing concentrations of the test compounds, and the pA_2 values were then calculated.

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The Output Mechanism of Circadian Pacemaker Neuropeptide PDF in the Regulation of Bimodal Locomotor Distribution

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Animals usually exhibit a bimodal morning and evening activity profile in the circadian cycle. Pigment-dispersing factor PDF is an 18-mer neuropeptide involved in the circadian output of insect locomotor activity. However, the mechanism how does PDF regulate such bimodal activities have not been well understood. We here propose a model output mechanism in relation to the circadian clock protein PERIOD isoforms which exhibit a bimodal expression.

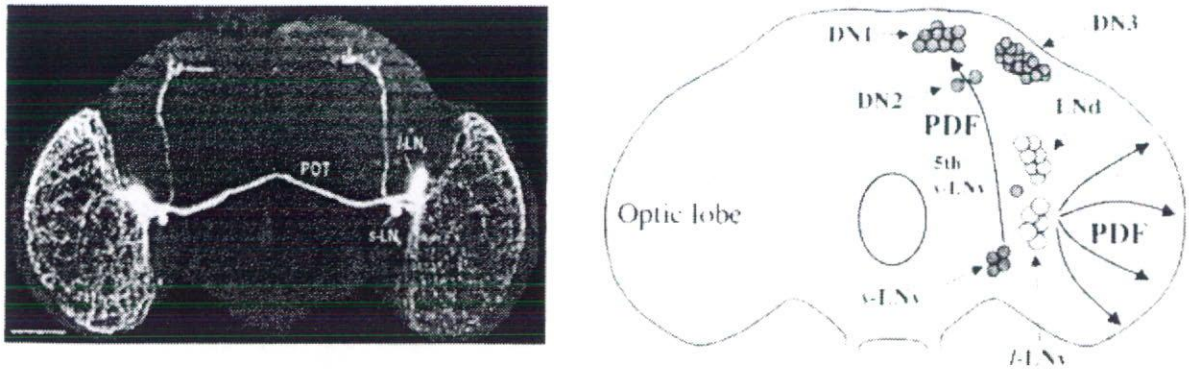
Keywords: circadian rhythm, evening oscillator, morning oscillator, *pigment-dispersing factor* (PDF), *period*

Introduction

Pigment-dispersing factor PDF is an 18-mer peptide involved in the circadian clock as a principal neuromodulator [1-3]. Its importance in transmitting the circadian rhythm of the locomotor activity is well acknowledged. The small (*s*-) and large (*l*-) LNv (ventral lateral neuron) uniquely express PDF that drives a rhythmic behavior especially under the constant darkness (Fig. 1). The protein PERIOD is an essential component in the circadian molecular mechanisms of the fruit fly *Drosophila melanogaster* (Fig. 2A). The circadian clock forms a transcriptional negative-feedback loop containing PERIOD as one of the clock protein components [4].

Animals usually exhibit a bimodal distribution of activities in the circadian cycle. The idea that two circadian oscillators exist individually has been proposed to explain such bimodal activity rhythms. In *Drosophila*, specific neuronal groups, namely the group of four *s*-LNv neurons and the group of LNd and 5th *s*-LNv, has been dedicated to morning and evening behaviors, respectively [5,6]. However, little is known about the molecular mechanisms of the oscillator systems present in these neurons.

We have hypothesized that the alternative splicing variants of PERIOD protein are required independently to drive the two oscillators, since the time-dependent expression and secretion of PDF in the LNv should be regulated by the two independent oscillators, involving individual PERIOD protein. We assumed the



PDF-expressing neurons	Projection
s-LNvs	Dorsal neuron
l-LNvs	Optic lobe

Fig. 1. PDF-expressing neuronal groups in the fly D. melanogaster

variant isoforms to be such key proteins. Then, we attempted to propose a model for the regulation of PDF expression and secretion by PERIOD bimodality. We here describe that *Drosophila* indeed has several different alternative splicing variants of *period* and the resulting PERIOD, both of which are in a bimodal distribution of expression day by day.

Results and Discussion

In the present study, for elucidation of the output mechanism of PDF, we first attempted to reveal the bimodal distributions of *period* mRNA in the fruit fly *D. melanogaster*. The circadian expression profiles of *period* mRNA were measured by

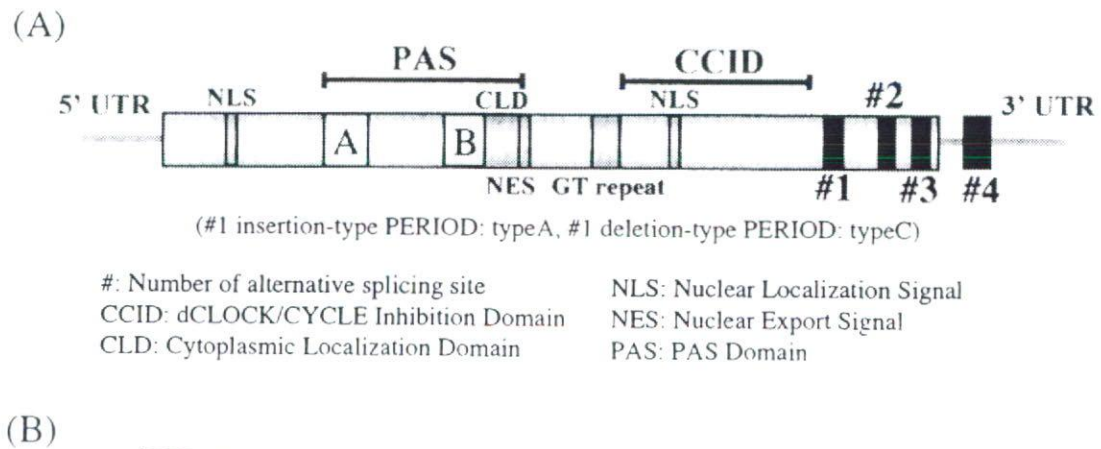


Fig. 2. Schematic structures of D. melanogaster *PERIOD and its isoforms*