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Ligand specificities of recombinant retinoic acid receptors RARα and RARβ

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Binding of retinoic acid (RA) to specific RA receptors α and β (RARα and RARβ) was studied. Receptors were obtained in two ways: (1) full-length receptors were produced by transient expression of the respective human cDNAs in COS 1 cells; and (2) the ligand-binding domains of RARα and RARβ were produced in *Escherichia coli*. RA binding to the wild-type and truncated forms of the receptor was identical for both RARα and RARβ, indicating that the ligand-binding domains have retained the binding characteristics of the intact receptors. Furthermore, RA bound with the same affinity to both RARα and RARβ. Only retinoid analogues with an acidic end-group were able to actively bind to both receptors. On measuring the binding of various retinoids, we have found that the properties of the ligand-binding sites of RARα and RARβ were rather similar. Two retinoid analogues were capable of binding preferentially to either RARα or RARβ, suggesting that it may be possible to synthesize specific ligands for RARα and RARβ.

INTRODUCTION

Retinoids, natural and synthetic analogues of vitamin A, exert strong effects on cellular growth and differentiation (Roberts & Sporn, 1984; Sherman, 1986; Orfanos *et al.*, 1987). Synthetic retinoids represent a class of compounds having therapeutic value in the treatment of dermatological disorders, such as acne and psoriasis. Moreover, retinoids may be of value as immunomodulators and as preventive and therapeutic anti-cancer agents (Bollag, 1979, 1983; Lippman *et al.*, 1987). Development of new retinoids faces the problems of pharmacological specificity and side-effects, particularly teratogenicity.

The mechanism of action of retinoids is still unclear. However, the existence of three receptors for retinoic acid (RARα, RARβ and RARγ) has been reported (Petkovich *et al.*, 1987; Giguère *et al.*, 1987; Benbrook *et al.*, 1988; Brand *et al.*, 1988; Zelent *et al.*, 1989). These receptors are nuclear regulators which control gene transcription. Studies on nuclear receptors have shown that two receptor functions, i.e. ligand binding and DNA binding, are fulfilled by two distinct and independent domains of the molecule. Upon ligand binding, RARs bind to specific DNA sequences and control the transcription of specific genes (Graupner *et al.*, 1989; Vasios *et al.*, 1989; De Thé *et al.*, 1990). This will result in increased and/or decreased synthesis of specific proteins leading to their eventual biological effects. The first step in retinoid action, i.e. receptor binding, is therefore of major importance in retinoid effects.

We have used recombinant RARα and RARβ to characterize the binding properties of retinoids. RA bound with the same affinity to both receptors. Only retinoids with a carboxylic end group were active ligands. Binding studies with selected retinoids strongly suggested that the physico-chemical properties of the ligand-binding sites of both receptors were very similar, but not identical.

EXPERIMENTAL

Materials

All-*trans*-RA and retinoid analogues were synthetized at Hoffmann–La Roche. Lipofectin was purchased from Bethesda Research Laboratories. All-*trans*-[^{3}H]RA (50 Ci/mmol) was obtained from New England Nuclear. Optimem 1 culture medium was from Gibco. The plasmids RARαO, ER-RARα.CAS and ER-RARβ.CAS were provided by Dr. P. Chambon (Faculté de Médecine, Strasbourg, France). All other chemicals were of the best grade commercially available.

Cell culture and cell transfection

COS 1 cells were grown attached to plastic dishes in Optimen 1 medium supplemented with 4% fetal calf serum. For transient expression of RARs, cells were replated at 80% confluence in 10 cm dishes 24 h before the experiment. The cells were transfected with 10 μg of plasmid DNA diluted in 20 μl of lipofectin (Felgner *et al.*, 1987), in 6 ml of Optimem 1 medium per 10 cm dish, for 18–20 h. Then 6 ml of fresh medium containing 8% fetal calf serum was added to the dish. At 24–48 h later, the cells were collected in phosphate-buffered saline (PBS) using a rubber policeman and washed once in cold PBS. Cell nuclear extracts were prepared as described by Nervi *et al.* (1989), with some modifications. Cell pellets from two or three dishes were resuspended in 0.3–0.6 ml of 5 mM-sodium phosphate buffer, pH 7.4, containing 10% glycerol, 10 mM-thioglycerol, 2 mM-phenylmethanesulphonyl fluoride (PMSF), aprotinin (1000 trypsin inhibitor units/ml) and leupeptin (10 μg/ml). The cells were disrupted by three cycles of freeze and thaw and centrifuged at 1000 *g* for 15 min at 4 °C in Eppendorf tubes. The pellets, containing the nuclei, were washed once in the same buffer and extracted in 0.5 ml of 10 mM-Tris/HCl buffer containing 0.8 M-KCl, 1.5 mM-EDTA, 10% glycerol, 10 mM-thioglycerol, 2 mM-

Abbreviations used: PBS, phosphate-buffered saline; DMSO, dimethyl sulphoxide; PMSF, phenylmethanesulphonyl fluoride; RA, retinoic acid; RAR, retinoic acid receptor; CRABP, cytoplasmic retinoic acid binding protein; IC_{50} concentration causing 50% inhibition of binding.

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PMSF, aprotinin (1000 trypsin inhibitor units/ml) and leupeptin (10 μg/ml) for 1 h at 4 °C, and then centrifuged at 130000 *g* for 30 min at 4 °C. The supernatants, referred to as nuclear extracts, were either used immediately or kept frozen at −80 °C before use.

Construction of the expression plasmids, and production and purification of the ligand-binding domains of RARα and RARβ

For the expression of the RA receptors in *Escherichia coli*, the expression vector pDS56/RBSII was used (Stüber *et al.*, 1990). This vector contains the nucleotide sequence coding for the translation-initiating methionine residue and the sequence coding for six consecutive histidine residues followed by a *Bam*HI site for insertion of the sequence to be expressed (the exact nucleotide sequences are shown below). This results in a protein having six histidine residues at its *N*-terminus which bind to nickel with high affinity even under strong denaturing conditions such as 6 M-guanidine/HCl or 8 M-urea. This nickel-binding property can be used for easy purification of the protein by binding to a Ni-chelate resin followed by elution with a low pH buffer (Hochuli, 1990). The human RARα cDNA (Petkovich *et al.*, 1987) was excised from the pSG1 vector with *Eco*RI and digested with *Hinf*1. The *Hinf*1-*Eco*RI fragment was rendered blunt-ended, followed by the addition of a 8-mer *Bgl*II linker, and was ligated into the *Bam*HI site of the *E. coli* expression vector pDS56/RSBII, 6 × His (Stüber *et al.*, 1990). This gave rise to the plasmid HRARα-(His)6.126. Plasmid HRARβ-ER.CAS (Brand *et al.*, 1988) was digested with *Xho*I and *Bam*HI. After filling the ends, 10-mer *Bam*HI linkers were added and ligated into the *E. coli* expression vector pDS56/RSBII, 6 × His, resulting in the plasmid HRARβ-(His)6.147. Nucleotide sequences from the junction, confirmed by sequence analysis, were as follows. HRARα-(His)6.126: ATGAGAGGATCGCATCACCATCACCATCACGCATCTGAG (126); HRARβ-(His)6.147: ATGAGAGGATCGCATCACCATCACCATCACGCATCCCGTCGAGAATCC (147). The plasmids HRARα-(His)6.126 and HRARβ-(His)6.147 were expressed on *E. coli* M-15 (Stüber *et al.*, 1990) at a level of about 5% of the total *E. coli* protein (as estimated by SDS/PAGE). Receptors were solubilized from the cells by lysozyme (2.8 mg/ml) treatment in 25 mM-Tris, (pH 8.0)/10 mM-EDTA/50 mM-glucose/2 mM-dithiothreitol (DTT) for 10 min at 22 °C. The digest was homogenized after the addition of NaCl (0.3 M), Triton X-100 (1%) and PMSF (1 mM) (final concentrations) in a Polytron at 4 °C (maximal speed, 2 min). Aggregates were centrifuged at 10000 *g* for 15 min. The supernatants contained about 50% of the total *E. coli* proteins, but only 1–5% of the receptors were present in the extract, suggesting that the majority of the receptors were in inclusion bodies and/or not properly folded. These supernatants, referred to as crude receptor extracts, were stored at −80 °C. Alternatively, for purification the cells were extracted in 6 M-guanidine/HCl/0.1 M-$NaHPO_4$, pH 8.0. Receptors were purified by applying the cleared lysate (10000 *g*, 10 min) to a Ni-chelate resin, to which they were bound via their hexa-His tag (Hochuli, 1990). The column was washed with 8 M-urea/0.1 M-$NaPO_4$/10 mM-Tris, pH 8.0, followed by the same solution at pH 6.3. Receptors were eluted by lowering the pH to 5.9 and 4.5. The yield was about 3 mg of receptors per litre of culture, with a purity of about 50%. The majority of the contaminating proteins were most probably degradation or premature termination products of the receptor that were co-purified via their hexa-His tag. The fractions containing the purified proteins were dialysed against a 1000-fold excess of PBS. Precipitated proteins were removed by centrifugation.

Retinoid-binding assay

In typical assays, 0.2–0.4 pmol of receptors were incubated in 8 mm × 40 mm glass microtubes (A. Riegger, Basle, Switzerland) in 0.2 ml of 50 mM-Tris/HCl (pH 7.4)/50 mM-NaCl/2 mM-EDTA (binding buffer) containing 0.5% gelatin, 1% dimethyl sulphoxide (DMSO), 2–5 nM-[^{3}H]RA and various concentrations of unlabelled retinoids. After 3–4 h of incubation at 22 °C, 0.15 ml of chilled charcoal/dextran suspension (5% Norit A/0.5% dextran T40 in binding buffer) were added for 15 min at 4 °C. The tubes were centrifuged at 12000 *g* for 15 min and the supernatants were counted for radioactivity. Binding parameters were calculated from competition curves using the 'Ligand' computer program (Munson & Rodbard, 1980). Non-specific binding measured in the presence of 1 μM unlabelled retinoic acid was 5–10% of total added counts.

Preparation of retinoid solutions

Retinoids were solubilized in DMSO as 1–10 mM stock solutions. Further dilutions were made in binding buffer containing 4% DMSO. Stock solutions were kept at −80 °C. Some retinoids were unstable upon storage and new solutions were prepared for each experiment.

Molecular mass determination.

Extracts containing receptors were incubated as for binding experiments with 300 nM-[^{3}H]RA for 2 h at 22 °C. Non-specific binding was measured in the presence of a 200-fold excess of unlabelled RA. Unbound ligand was removed with charcoal/dextran and the extracts were fractionated on a Superose 12HR 10/30 column at a flow rate of 0.8 ml/min using 50 mM-Tris/HCl (pH 8.5)/2 mM-EDTA/1 mM-PMSF/10 mM-thioglycerol/10% glycerol/0.4 M-KCl as eluant (Nervi *et al.*, 1989). Fractions of 0.27 ml were collected and radioactivity was measured.

Toxic effects of retinoids *in vivo*

Retinoids were administered intraperitoneally, daily (weekdays) for 2 weeks to outbred female Ibm: Moro (SPF) mice weighing 26–30 g. Body weight development, hair loss, changes in the mucosa of the nose and mouth and bone fractures were recorded. These side-effects usually appeared after 5–7 days of treatment and were rated using a scale of 0–4 (Teelmann, 1990). Hypervitaminosis A was defined as that condition of the animals where the sum of the side-effects graded at least 3.

RESULTS

RA binding to full-length and ligand-binding domains of RARα and RARβ

The full-length receptors were produced by transient expression of receptor plasmids in COS cells. Cell nuclei were prepared and receptors were extracted using high salt concentrations (Nervi *et al.*, 1989). As shown in Fig. 1, nuclear extracts obtained from cells transfected with RARαO (human RARα gene) and ER-RARβ.CAS (human chimeric RARβ gene) showed specific binding of RA, whereas extracts from control non-transfected cells had no binding activity. As determined by Scatchard analysis, the amount of receptors obtained by cell transfection was, however, relatively low (10–60 fmol/mg of total cell proteins) for retinoid screening purposes. For this reason, another source of receptors had to be found. Several studies on nuclear steroid receptors have reported that ligand binding requires only the *C*-terminal portion of the protein, called the ligand-binding domain. The putative ligand-binding domains of RARα and RARβ (262 and 255 amino acids respectively) were therefore cloned and expressed in *E. coli*. Crude

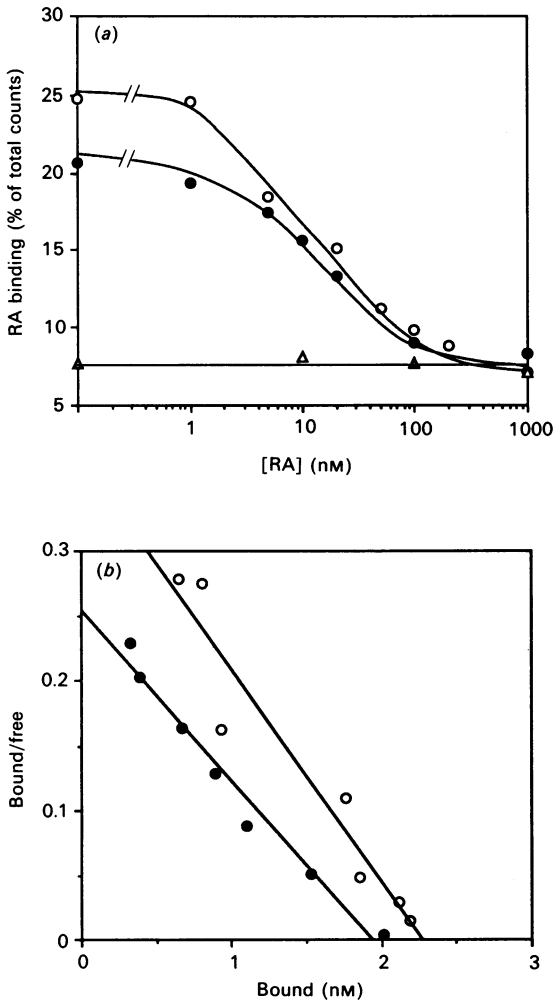


Fig. 1. RA binding to human RARα and RARβ

(*a*) Nuclear extracts of control untransfected COS cells (△) and cells transfected with RARα (○) and RARβ (●) genes were incubated with 2 nM-[^{3}H]RA and the indicated concentrations of unlabelled ligand for 3 h at 22 °C. The percentage of [^{3}H]RA bound is plotted as a function of RA concentration. (*b*) Scatchard plots of the data in (*a*).

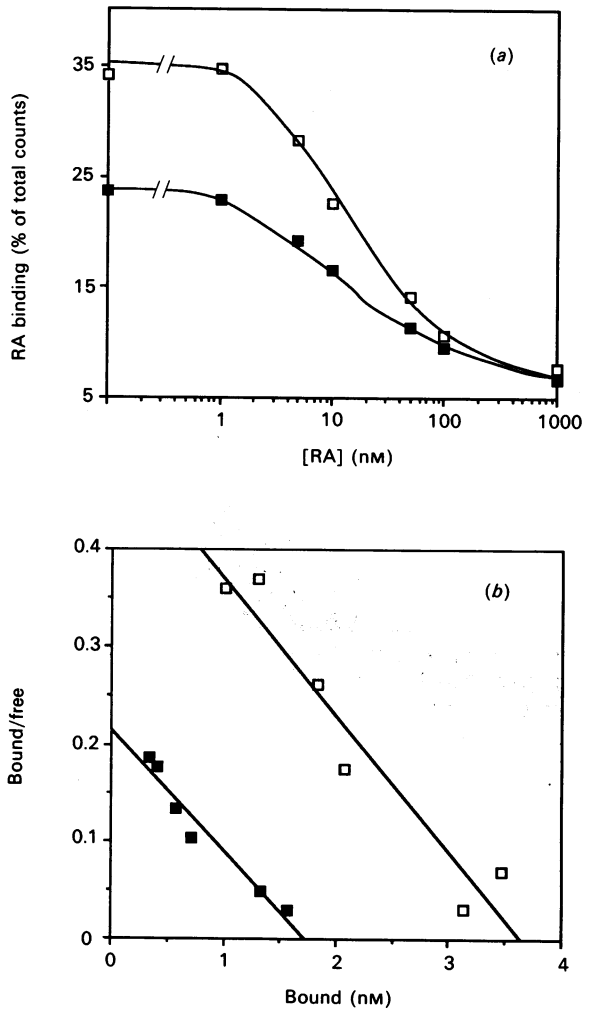


Fig. 2. RA binding to the ligand-binding domain of human RARα and RARβ

(*a*) *E. coli* extracts containing the ligand-binding domain of human RARα (□) and RARβ (■) were incubated as described in the legend to Fig. 1. The percentage of [^{3}H]RA bound is plotted as a function of RA concentration. (*b*) Scatchard plots of the data in (*a*).

bacterial extracts and purified receptors were used for binding studies. As no difference in RA binding was observed between purified and crude receptor preparations (e.g. K_d values for RARα were 2–10 nM and 3–12 nM respectively), most of our measurements were made on crude bacterial extracts. As depicted in Fig. 2, extracts containing the ligand-binding domains of either RARα of RARβ were active in binding labelled RA, and this binding was competed for by increasing concentrations of unlabelled ligand. Binding competition curves for the full-length receptors and the truncated receptor ligand-binding domains were analyzed using 'Ligand' computed fitting (Munson & Rodbard, 1980). The derived Scatchard plots are shown in Fig. 1(*b*) and 2(*b*), and the equilibrium binding constants are summarized in Table 1. In these experiments, receptor concentrations were 2–4 nM and non-specific binding was 7–9% of the total added radioactivity. All of the Scatchard plots were linear, as expected for receptors with a single type of binding site. In addition, the dissociation constants (K_d) of RA were similar for all types of receptors. These results indicated, first, that both RARα and RARβ bound RA with the same affinity, and secondly, that the ligand-binding domains of the receptors have

Table 1. Equilibrium parameters of RA binding to the full-length receptors and the ligand-binding domains of RARα and RAR β

COS, full-length receptors expressed in COS cells; rRAR, truncated receptors (ligand-binding domains) expressed in *E. coli*. Values (means ± S.E.M.) were derived by computer analysis of the binding data represented in Figs. 1 and 2.

Receptor	K_d (nM)	IC_{50} (nM)	Hill coefficient
RARα (COS)	6.1 ± 1.9	11.5	0.8
RARβ (COS)	7.6 ± 1.9	11.6	1.2
rRARα	6.2 ± 1.5	14.4	1.0
rRARβ	8.0 ± 2.1	16.6	0.8

retained the full binding properties of the native receptors. This last observation was strengthened by the fact that RA binding to bacterial extracts was highly specific. Retinol and retinal, two natural retinoids, had little or no effect in competing for binding when compared with RA (Table 2). In addition, other ligands

Table 2. Binding specificity of rRARα and rRARβ

Receptors were incubated with 2 nM labelled RA and various additions for 18 h at 4 °C.

Addition	Binding (%) rRARα	rRARβ
None	100	100
RA (1 μM)	10	8
Retinol (1 μM)	75	70
Retinal (1 μM)	76	107
Dexamethasone (10 μM)	92	82
β-Oestradiol (10 μM)	92	89
Progesterone (10 μM)	85	103

for nuclear receptors, such as dexamethasone, oestradiol and progesterone, did not significantly bind to RARα or RARβ (Table 2).

Characterization and determination of the molecular masses of the various receptors preparations

[^{3}H]RA-labelled receptors were analysed by h.p.l.c. on a size-exclusion Superose column. The h.p.l.c. profile of RARα showed a single peak of radioactivity, which was abolished when the receptors were incubated with an excess (200-fold) of unlabelled RA (Fig. 3*a*). It should be noted that the molecular mass of the RA receptor is clearly distinct from that of cytosolic retinoic acid binding protein (CRABP), which is undetectable in the nuclear fraction of the COS cells (Figs. 3*a* and 3*d*). Bacterial extracts containing the ligand-binding protein of human RARα and RARβ (referred to as rRARα and rRARβ) yielded a single peak specifically labelled with [^{3}H]RA (Figs. 3*b* and 3*c*). The peak which was eluted at fractions 11–15 was non-specific and was eluted in the void volume of the column. The apparent molecular masses calculated from the elution times were 48 kDa, 30 kDa and 28 kDa for RARα, rRARα and rRARβ respectively (Fig. 3*d*). These values were in good agreement with the molecular masses calculated from the amino acid sequences.

Relationships between structure and binding of retinoids

The relative receptor binding activity of several retinoids is reported in Tables 3 and 4. Modifications of the cyclohexenyl ring resulted in a marked loss of retinoid binding. For instance, 4-oxo-RA (Table 3, compound 8), a metabolite of RA (Frolik *et al.*, 1979, 1980), retained some binding activity, whereas the other RA metabolite, 4-hydroxy-RA (Table 3, compound 12), was inactive. The RA analogue with an aromatic ring (Table 3, compound 10) was also poorly active. 13-*cis*-RA had a decreased ability to bind to both receptors (Table 3, compound 7). Saturation of the side-chain double bond at positions 7 and 8

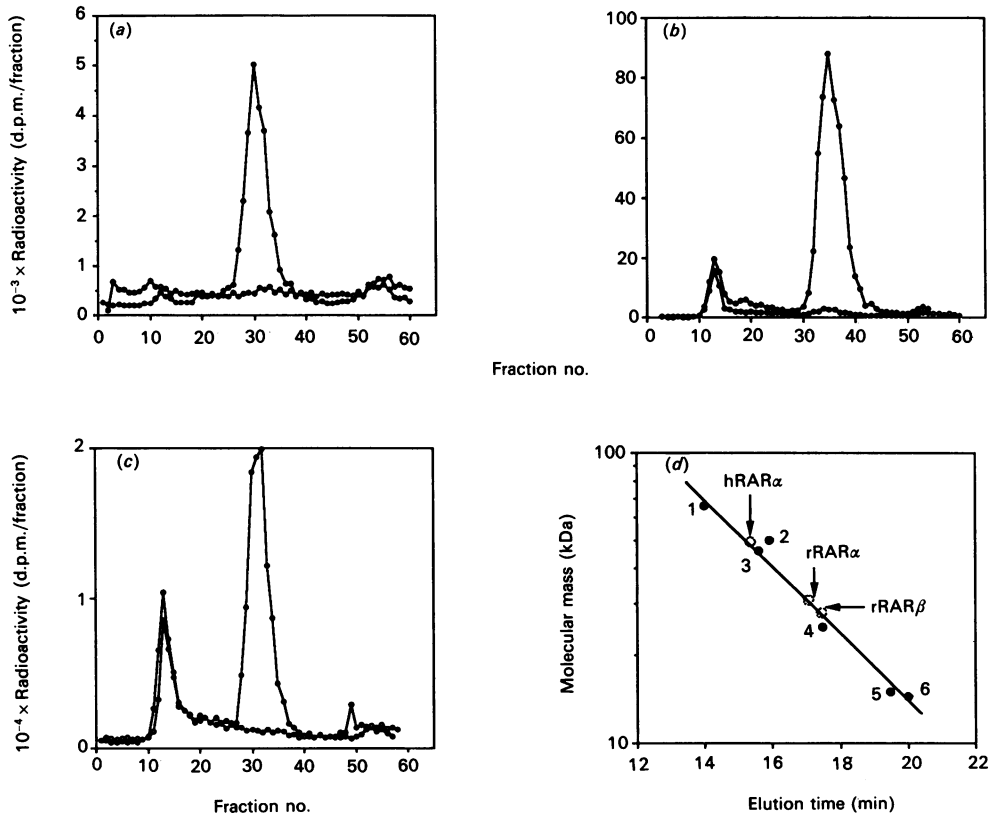
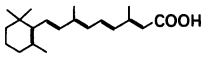
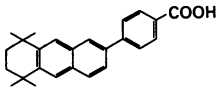
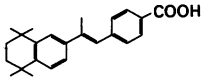
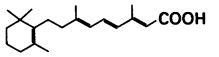
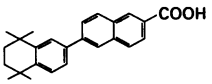
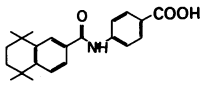
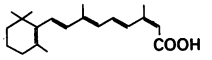
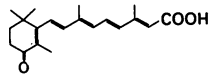
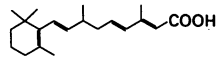
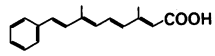
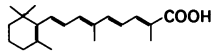
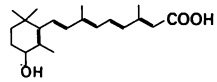
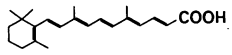


Fig. 3. H.p.l.c. elution profile of [^{3}H]RA binding to various RARs preparations

Nuclear extracts of RARαO-transfected COS cells (*a*), and bacterial extracts containing the ligand-binding domains of human RARα (*b*) and RARβ (*c*) were incubated as described in the legend to Fig. 1. The samples were fractionated over a Superose 12HR 10/30 column and radioactivity was counted in each fraction (0.27 ml). ●, [^{3}H]RA (300 nM) only; ○, [^{3}H]RA and a 200-fold excess of unlabelled RA. (*d*) Molecular mass determination: the elution time of standard proteins is plotted as a function of their molecular mass. Elution times of HRARα were calculated from the data in (*a*), (*b*) and (*c*). Molecular mass standards: 1, human serum albumin; 2, immunoglobulin γ-chain; 3, ovalbumin; 4, chymotrypsin; 5, human CRABP; 6, cytochrome *c*.

Table 3. Binding to rRARα and rRARβ and biological activity of several retinoids

Relative receptor binding is defined as the ratio of the IC_{50} (retinoid concentration to inhibit 50% of specific labelled RA binding) for RA to that of the novel compound, expressed as a percentage. Relative effect is the ratio of ED_{50} (retinoid concentration to produce half-maximal effect) for RA to that of the retinoid analogue. ND, not determined.

Compound no.	Name or Roche number	Chemical structure	Relative receptor binding (%)		Hypervitaminosis A (relative effect)
			rRARα	rRARβ	
1.	RA		100	100	1
2.	Ro 40-6976		140	280	8000
3.	Ro 13-7410 (arotinoid, TTNPB)		50	70	800
4.	Ro 11-0874		30	28	0.4
5.	Ro 19-0645 (TTNN)		3	33	ND
6.	Ro 40-6055 (Am 580)		21	0.5	0.3
7.	Ro 04-3780 (13-*cis*-RA)		15	19	0.2
8.	Ro 11-4824 (4-*oxo*-RA)		5	3	0.4
9.	Ro 11-1813		0.8	0.5	0.2
10.	Ro 08-8717		0.7	0.5	< 0.2
11.	Ro 10-0191		0.5	0.7	0.2
12.	Ro 10-2655 (4-hydroxy-RA)		0	0	0.8
13.	Ro 11-0976		0	0	0.8

(Table 3, compound 4) had a minor consequence on binding, while saturation at positions 9 and 10 (Table 3, compound 9) decreased markedly the binding ability of the analogue. A change in position of the two methyl groups on the side chain (Table 3, compound 11) also produced a decrease in binding. Elongation of the side chain resulted in a total loss of binding activity (Table 3, compound 13). In contrast, insertion of side chain double bonds into aromatic rings (Table 3, compounds 2 and 3) gave retinoid analogues with a binding affinity comparable with that of RA. Fig. 4 depicts the correlation of binding to RARα and RARβ for the retinoids reported in Table 3. The IC_{50} values (concentrations inhibiting 50% of [^{3}H]RA binding) were highly correlated, except for two retinoids. One analogue (Table 3, compound 5) had 10-fold higher affinity for RARβ than for RARα, whereas another (Table 3, compound 6) had a 40-fold higher affinity for RARα than for RARβ.

The ability of these retinoids to bind to RARα and RARβ has been compared with their toxic effects *in vivo* related to hypervitaminosis A. This involved a variety of side-effects, such as weight and hair loss, skin scaling and bone fracture (Teelmann 1989, 1990). As reported in Table 3, the higher the ability of binding of retinoids, the worse the side-effects. However, the correlation between receptor binding and side-effects was not complete. Some retinoids were much more toxic than RA, but their binding affinities were slightly higher or lower (Table 3, compounds 2 and 3). Other retinoids were biologically active even in the absence of receptor binding, indicating other mechanisms of retinoid action or activation.

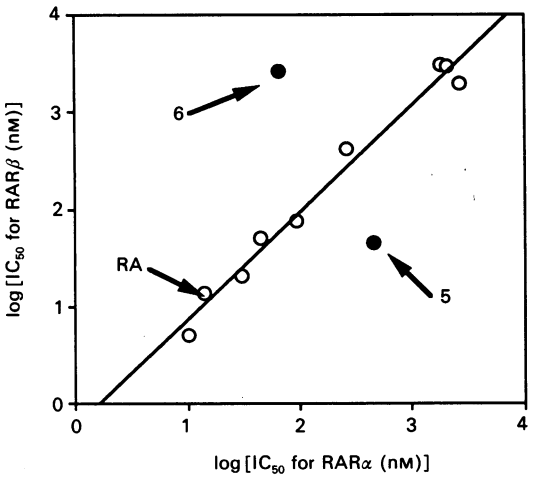
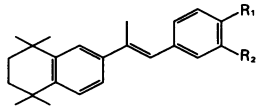


Fig. 4. Binding comparison of various retinoids to RARα and RARβ

The IC_{50} of retinoids depicted in Table 3 for RARα are plotted as a function of those for RARβ. 5 and 6 are numbers which refer to the numbers in Table 3.

Table 4. Effect of polar end-group modification on retinoid binding to rRARα and rRARβ

Relative receptor binding is defined as the ratio of the ED_{50} for TTNPB (Ro 13-7410) to that of the novel compound, expressed as a percentage. The structure of the compound used was as follows:



Compound type no.	R_1	R_2	Relative receptor binding (%) rRARα	rRARβ
1.	COOH	H	100	100
	SO_2^- Na^+	H	10	18
	PO_3H_2	H	0.4	0.6
2.	H, OH F, I, Br, Cl CH_3, CF_3 $CH(CH_3)_2$ $C(CH_3)_3$ NO_2, NH_2 SO_2CH_3, $SO_2C_2H_5$	H	0	0
3.	H	COOH	0	0.3
	H	PO_3H_2	0	< 0.3

The effect of polar end-group modifications on retinoid binding is reported in Table 4. Various analogues of the arotinoid Ro 13-7410 have been studied, since this compound bound with high affinity to both receptors (Table 3, compound 3). The presence of a free terminal carboxy function was a prerequisite for high retinoid binding. Replacement of the carboxy group with a sulphonyl or phosphonyl group produced a dramatic drop in binding (Table 4, compound 1). Any other substitution on the arotinoid resulted in a total loss of the binding ability of the retinoid (Table 4, compound 2). Carboxy esters did not bind to either receptor type (results not shown). Moreover, the position of the carboxy end group appeared to be important, since moving it from the *para* to the *meta* position also caused a total loss of activity (Table 4, compound 3).

DISCUSSION

Several genes for human RARs have been isolated (Petkovich *et al.*, 1987; Giguère *et al.*, 1987; Benbrook *et al.*, 1988; Brand *et al.*, 1988) and appear to be co-expressed in a large variety of tissues (Nervi *et al.*, 1989; Hashimoto *et al.*, 1989, 1990; Rees & Redfern, 1989). This heterogeneity of RARs poses two important questions: do these receptors have different ligand specificities, and do they regulate the expression of different genes? RARs are, however, proteins of low abundance (a few thousand receptors per cell) and purification of these proteins from natural sources in quantities sufficient to address these questions would be difficult and tedious. In order to overcome these difficulties, we have produced RARs by genetic engineering.

Initial attempts to produce full-length RARα and RARβ in *E. coli* gave only very low expression levels (results not shown). Since RARs belong to the steroid/thyroid receptor family, which have been shown to be composed of various independently functional domains (Giguère *et al.*, 1986; Kumar *et al.* 1987; Evans, 1988), we expressed only the putative ligand-binding domains. These *C*-terminal parts of RARα and RARβ (262 and 255 amino acids respectively) gave good expression levels in *E. coli* and were therefore used for the subsequent binding studies.

In order to prove that the receptors produced in *E. coli* were adequate for binding studies, we also measured binding to the full-length receptors. Full-length RARs were obtained by transient expression of human RAR genes in eukaryotic COS 1 cells. The receptors were prepared from nuclear extracts, which had the benefit of removing any contaminating cytoplasmic retinoid binding proteins (CRBP and CRABP) (Fig. 3*a*). When retinoic acid binding to the expressed ligand-binding domains was compared with the binding obtained with the full-length receptors, the measured affinities for RA were identical. Furthermore, there was a good correlation between the binding properties of different retinoid analogues and their transactivation activity in a transient transfection assay (results not shown). This clearly demonstrates that the ligand-binding domains have retained the complete binding properties of the entire receptors.

The K_d values of RA for RARα and RARβ were identical, indicating similar binding affinity of both receptors for RA. From our studies with different RA analogues, it appears that the ligand-binding sites of both receptors are very similar in nature. We have studied the binding of retinoid analogues which differ from RA by single molecular modifications. Any saturation of the side-chain double bonds, any elongation of the side-chain, or any cyclohexenyl ring alteration resulted in retinoid analogues with lower binding affinities than RA. These decreases in binding were identical for RARα and RARβ, suggesting similar physico-chemical environments of the binding sites. However, the RARα and RARβ binding sites were not completely identical. The arotinoid Ro 40-6055, also known as Am 580 (Jetten *et al.*, 1987), bound 40-fold better to RARα than to RARβ. In contrast, another retinoid, Ro 19-0645, preferentially bound to RARβ. Although the nature of the difference between the two binding sites is still unclear, it indicates the possibility of developing specific ligands for RARα and RARβ.

The comparison between the binding results and biological effects show that other properties of the RA analogues are also important. For instance, the well-studied arotinoid Ro 13-7410 has only 50–70 % of the binding activity of RA, yet is nearly 1000-fold more potent than RA in inducing hypervitaminosis A (Table 3), preventing chondrogenesis in rat limb bud cells and producing teratogenicity in mice (Kistler, 1987). This is probably due to the

high stability of the compound *in vivo*. It is also possible that the arotinoid is more potent than RA in activating receptor function. Another interesting observation is that some retinoid analogues have biological effects in the absence of receptor binding. For instance, retinol, retinal and retinyl esters do not bind significantly to RARα and RARβ (Tables 2 and 4), even though they have retinoid-like activities. These retinoids must be metabolized by the cells into active binding molecules. It is well established that retinol and retinal can be oxidized into RA and that cellular esterases can transform retinyl esters in their active binding acid forms. In addition, RA can replace retinol for most functions, except in vision and in male fertility.

4-Hydroxy-RA, one of the end-products of RA metabolism, and the longer side-chain analogue Ro 11-0976 have biological effects, such as production of hypervitaminosis A (Table 4), control of keratinization of the hamster tracheal organ in culture (Sporn & Roberts, 1984) and inhibition of limb bud cell differentiation (Kistler, 1987), although they do not bind to the receptors in our assays. One possibility is that their biological actions involve binding to other RARs, such as RARγ or other, as yet unidentified, receptors. However, these compounds could act through different mechanisms not involving nuclear receptors. Retinoid action on protein secretion has been observed in the absence of the nucleus (Bolmer & Wolf, 1982). Membrane enzyme activities appeared to be modified by retinoids within minutes, excluding action on gene transcription (Johnson & Davies, 1986; Lochner *et al.*, 1986; Smith *et al.*, 1989).

In conclusion, our results show that binding studies with RA analogues and recombinant RARs can provide information about the ligand specificities of the receptors. This in turn may lead to the development of synthetic retinoids having increased RAR subtype specificities and therefore greater tissue specificities and lower toxicities.

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REFERENCES

Benbrook, D., Lernhardt, E. & Pfahl, M. (1988) Nature (London) **333**, 669–672
Bollag, W. (1979) Cancer Chemother. Pharmacol. **3**, 207–215
Bollag, W. (1983) Lancet **i**, 860–863
Bolmer, S. D. & Wolf, G. (1982) Proc. Natl. Acad. Sci. U.S.A. **79**, 6541–6545
Brand, N. J., Petkovich, M., Krust, A., Chambon, P., De Thé, H., Marchio, A. Tiollais, P. & Dejean, A. (1988) Nature (London) **332**, 850–853
De Thé, H., Vivancoruiz, M. D., Tiollais, P., Stunnenberg, H. & Dejean, A. (1990) Nature (London) **343**, 177–180
Evans, R. M. (1988) Science **240**, 889–895
Felgner, P. L., Gadek, T. R., Holm, M., Roman, R., Chan, H. W., Wenz, M. Northrop, J. P., Ringold, G. M. & Danielsen, M. (1987) Proc. Natl. Acad. Sci. U.S.A. **84**, 7413–7417
Frolik, C. A., Roberts, A. B., Tavela, T. E., Roller, P. P. & Newton, D. L. (1979) Biochemistry **18**, 2092–2097
Frolik, C. A., Roller, P. O., Roberts, A. B. & Sporn, M. B. (1980) J. Biol. Chem. **225**, 8057–8062
Giguère, V., Hollenberg, S. M., Rosenfeld, M. G. & Evans, R. M. (1986) Cell **46**, 645–652
Giguère, V., Ong, E. S., Segui, P. & Evans, R. M. (1987) Nature (London) **330**, 624–629
Graupner, G., Wills, K. N., Tzukerman, M., Zhang, X.-K. & Pfahl, M. (1989) Nature (London) **340**, 653–656
Hashimoto, Y., Petkovich, M., Gaub, M. P., Kageshika, H., Shudo, K. & Chambon, P. (1989) Mol. Endocrinol. **3**, 1046–1052
Hashimoto, Y., Kagechika, H. & Shudo, K. (1990) Biochem. Biophys. Res. Commun. **166**, 1300–1307
Hochuli, E. (1990) in Genetic Engineering, Principles and Methods (Setlow, J. K., ed.), pp. 171–193, Plenum Press, New York
Jetten, A. M., Anderson, K., Deas, M. A., Kagechika, H., Lotan, R., Rearick, J. I. & Shudo, K. (1987) Cancer Res. **47**, 3523–3527
Johnson, J. D. & Davies, P. J. A. (1986) J. Biol. Chem. **261**, 14982–14986
Kistler, A. (1987) Arch. Toxicol. **60**, 403–414
Kumar, V., Green, S., Stack, G., Berry, M., Jin, J.-R. & Chambon, P. (1987) Cell **51**, 941–951
Lippman, S. M., Kessler, J. F. & Meyskens, F. L. (1987) Cancer Treat. Rep. **71**, 391–405
Lochner, J. E., Badwey, J. A., Horn, W. & Karnovsky, M. L. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 7673–7677
Munson, P. J. & Rodbard, D. (1980) Anal. Biochem. **107**, 220–239
Nervi, C., Grippo, J. F., Sherman, M. I., George, M. D. & Jetten, A. M. (1989) Proc. Natl. Acad. Sci. U.S.A. **86**, 5854–5858
Orfanos, C. E., Ehlert, R. & Gollnick, H. (1987) Drugs **34**, 459–503
Petkovich, M., Brand, N. J., Krust, A. & Chambon, P. (1987) Nature (London) **330**, 444–450
Rees, J. L. & Redfern, P. F. (1989) J. Invest. Dermatol. **93**, 818–820
Roberts, A. B. & Sporn, M. B. (1984) in The Retinoids (Sporn, M. B., Roberts, A. B. & Goodman, D. S., eds.), vol. 2, pp. 209–286, Academic Press, New York
Sherman, M. I. (1986) in Retinoids and Cell Differentiation (Sherman, M. I., ed.), pp. 161–186, CRC Press, New York
Smith, T. J., Davies, F. B. & Davies, P. J. (1989) J. Biol. Chem. **264**, 687–689
Sporn, M. B. & Roberts, A. B. (1984) in The Retinoids (Sporn, M. B., Roberts, A. B. & Goodman, D. S., eds.), vol. 1, pp. 236–275, Academic Press, New York
Stüber, D., Matile, H. & Garotta, G. (1990) Immunol. Methods, in the press
Teelmann, K. (1989) Pharmacol Ther. **40**, 19–43
Teelmann, K. (1990) Methods Enzymol. **190**, 382–395
Vasios, G. W., Gold, J. D., Petkovich, M., Chambon, P. & Gudas, L. J. (1989) Proc. Natl. Acad. Sci. U.S.A. **86**, 9099–9103
Zelent, A., Krust, A., Petkovich, M., Kastner, P. & Chambon, P. (1989) Nature (London) **339**, 714–717

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