Stereochemical requirements for the modulation by retinoic acid of thyroid hormone activation of Ca^{2+} -ATPase and binding at the human erythrocyte membrane

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Physiological concentrations of retinoic acid can block the activation of human erythrocyte Ca^{2+} -ATPase *in vitro* by thyroid hormone [Smith, Davis & Davis (1989) J. Biol. Chem. **264**, 687–689]. The present studies were undertaken to ascertain the nature of this blockade. Two binding sites for L-thyroxine (T_4) were demonstrated on washed erythrocyte membranes; the high-affinity site had a K_d value of 2.7×10^{-10} M and a B_{max} of 76 fmol/mg of protein. The lower-affinity site possessed a K_d of 1×10^{-8} M. Retinoic acid was as potent a displacer of radiolabelled T_4 as was the unlabelled hormone. Certain retinoic acid analogues with either ring or fatty acid side chain modifications retained some ability to displace [^{125}I] T_4 binding and to block iodothyronine activation of Ca^{2+} -ATPase. The side chain terminal carboxyl group was essential for full activity of the retinoic acid molecule. Its absence or replacement with an ethylsulphone group rendered the molecule considerably less active in the ATPase model. Retinol, 13-*cis*-retinoic acid, benzene-substituted all-*trans*-retinoic acid and polyprenoic acid all failed to influence iodothyronine binding or to block activation of Ca^{2+} -ATPase by T_4 . There was good agreement between the ability of an analogue to displace [^{125}I]iodothyronine binding and its ability to inhibit the T_4 -dependent activation of the Ca^{2+} -ATPase. It would appear from these observations that retinoic acid can modulate the activation of erythrocyte membrane Ca^{2+} -ATPase by thyroid hormone through a mechanism which involves displacement of iodothyronine from binding sites. These activities apparently derive from both the ring structure and the fatty acid side chain of the retinoic acid molecule.

INTRODUCTION

Thyroid hormones [3,5,3'-L-tri-iodothyronine (T_3) and L-thyroxine (T_4)] and the retinoids [retinoic acid (RA) and related compounds] represent two structurally diverse classes of regulatory molecules. The crucial discovery that the *c-erb A* proto-oncogene encodes a nuclear T_3 receptor [1,2] and shares 62% sequence identity with the RA receptor in the cysteine-rich DNA binding domain [3,4] has prompted inquiry into the possible overlap of the gene response networks for the two molecules. The RA receptor can bind to a thyroid hormone response element with high affinity and activate gene expression from promoters fused to this element [5]. While the actions of T_3 and RA at the genomic level have been characterized extensively, the nature of their direct effects on the plasma membrane remains to be elucidated.

Human erythrocytes are a useful model for studying metabolic events which do not involve genomic function, as they are enucleate at maturity. The recent observation that the enzymic expression of the Ca^{2+} pump, i.e. the Ca^{2+} -stimulable, Mg^{2+} -dependent ATPase (Ca^{2+} -ATPase; EC 3.6.1.3), is regulated by thyroid hormone [6–9] has provided an ideal opportunity for examining the direct action of thyroid hormones on the plasma membrane and for recognizing those factors which might influence extranuclear thyroid hormone action.

Rat and human red cell membranes contain high-affinity binding sites for thyroid hormone, and the nature of these determinants is apparently different in the two species [10–12]. There is reason to suspect that the plasma membrane is a primary site of thyroid hormone action, a notion which has been

explored in other cell types, including rat thymocytes [9,13]. Evidence for direct membrane actions of RA and related compounds derives from relatively few observations [14].

We have reported that RA partially blocked the activation *in vitro* of human red cell Ca^{2+} -ATPase by T_4 and T_3 [15]. The present paper describes studies which examine the structure-activity relationships of retinoid action on T_4 stimulation of Ca^{2+} -ATPase. We also report that RA and some of its analogues can displace radiolabelled iodothyronine from erythrocyte membrane binding sites in a manner which correlates well with the retinoid blockade of enzyme activation by thyroid hormone.

MATERIALS AND METHODS

T_4 , Na_2ATP , retinol, all-*trans*-RA and 13-*cis*-RA were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Ro 1-5488 (all-*trans*-RA), Ro 4-3780 (13-*cis*-RA), Ro 10-1670 (free acid of etretinate), Ro 13-7652 (13-*cis*-etretinate), Ro 12-7310 (an etretinate metabolite with a hydroxy group substituted for the methoxy group), Ro 13-7410 (arotinoid, free acid), Ro 15-0778 (arotinoid lacking a polar end group), Ro 15-1570 (arotinoid ethylsulphone), E-5166 (polyprenoic acid) and Ro 8-8717 (benzene-ring-substituted all-*trans*-RA) were kindly provided by Dr. S. Shapiro, Hoffmann-La Roche Inc. (Nutley, NJ, U.S.A.). [^{125}I] T_4 (1250 $\mu\text{Ci}/\mu\text{g}$), [^{125}I] T_3 (1000 $\mu\text{Ci}/\mu\text{g}$) and all-*trans*-[^3H]RA (50 Ci/mmol) were purchased from New England Nuclear (Boston, MA, U.S.A.).

Red cell membrane preparation

Fresh whole blood was obtained from fasted, healthy volunteers. Red cell membrane ghosts were prepared hypo-

Abbreviations used: T_3 , 3,5,3'-L-tri-iodothyronine; T_4 , 3,5,3',5'-L-tetraiodothyronine (thyroxine); RA, retinoic acid; Ca^{2+} -ATPase, Ca^{2+} -stimulable, Mg^{2+} -dependent ATPase.

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Fig. 1. The effect of unlabelled T_4 (●) and RA (○) on the binding of $[^{125}I]T_4$ (a), or of T_3 (●) and RA (○) on the binding of $[^{125}I]T_3$ (b) to human erythrocyte membranes

Each data point represents the mean of three experiments, each performed in duplicate. The insets are Scatchard analyses which demonstrate two binding sites each for T_4 and T_3 : (a) $y = 0.140 - 0.037x$; $y = 0.099 - 0.001x$; (b) $y = 0.180 - 0.022x$; $y = 0.125 - 0.001x$. B/F = bound/free.

osmotically as described previously [6] and washed membranes were stored at -70°C until assayed.

Radioligand binding studies

To determine whether RA, retinol and retinoid analogues affected binding of T_4 to red cell membranes, washed membranes [6] (0.5 mg/tube) were incubated with $[^{125}I]T_4$ (4.28 pM) in a buffer containing 10 mM-Tris (pH 7.4) and increasing concentrations of unlabelled T_4 , retinol, retinoic acid or the other retinoids tested. The reaction was carried out in a final volume of 1 ml for 60 min at 37°C . Appropriate diluent controls were included in each experiment. The separation of bound from unbound ligand was accomplished by centrifugation at $20000g \times 10$ min, followed by one wash with 1 ml of 10 mM-Tris at 4°C and recentrifugation. Studies were performed with duplicate samples. Total T_4 binding to membranes was $22.0 \pm 0.2\%$ of tracer T_4 ; specific binding was determined by

Fig. 2. Effect of increasing concentrations of all-*trans*-RA (Ro 1-5488) and seven retinoid analogues on $[^{125}I]T_4$ (4.3×10^{-12} M) binding to human erythrocyte membranes

Also shown is the effect of unlabelled T_4 in the same concentrations on displacement of tracer from membranes. Each data point represents the mean $\pm$ S.E.M. of results from three experiments, each performed in duplicate. Specific T_4 binding was calculated as total T_4 binding minus that found in the presence of 10^{-6} M unlabelled T_4 . Results are expressed as percentages of control specific tracer T_4 binding, which occurred in the absence of retinoid analogue or unlabelled hormone. C, control.

adding $1 \mu\text{M}$ unlabelled T_4 to selected samples, and accounted for 56 % of the total. The effect of retinoids on membrane binding of T_4 is expressed in the present studies as a percentage of control specific binding of tracer T_4 in the absence of added hormone or retinoid. Studies of radiolabelled T_3 and RA binding to red cell membranes were carried out in a similar manner.

Ca^{2+} -ATPase assay

Membranes were preincubated at 37°C for 1 h before assay in the presence of T_4 , retinoid or diluent (1 % ethanol). Enzyme activity was assayed as reported previously [6] and was taken as the difference in Na_2ATP hydrolysis in the presence and absence of $20 \mu\text{M}$ free Ca^{2+} . Enzyme activity was determined as μmol of P_i liberated/90 min per mg of membrane protein; the mean enzyme activity in these studies was 0.511 ± 0.028 (mean $\pm$ S.E.M.). T_4 (0.1 nM) increased enzyme activity by $0.181 \pm 0.029 \mu\text{mol}$ of P_i/mg of protein, which represented a 35 % increase in Ca^{2+} -ATPase activity. This T_4 concentration is associated with maximal enzyme stimulation [6]. The effect of retinoids on T_4 -stimulable enzyme activity is expressed in the present studies as a percentage of control hormone-stimulable Ca^{2+} -ATPase activity observed in the absence of retinoid analogue. The Ca^{2+} -ATPase activity was linear from 15 to 90 min in the presence and absence of RA.

Other methods

Protein content was determined by the method of Lowry *et al.* [16] using BSA as standard. Individual samples were assayed in

Table 1. Structure-activity relationships of retinoid analogues

Ro no.	Name	Basic chemical structure	T ₄ -stimulable Ca ²⁺ -ATPase activity (% of control)		Specific T ₄ binding to red cell membranes (% of control)	
			10 ⁻¹⁰ M	10 ⁻⁸ M	10 ⁻¹⁰ M	10 ⁻⁸ M
1-5488	all- <i>trans</i> -RA (tretinoin)		73 ± 3	46 ± 3	89 ± 7	54 ± 4
4-3780	13- <i>cis</i> -RA (isotretinoin)		97 ± 6	94 ± 6	96 ± 1	93 ± 2
E-5166	Polyprenoic acid		95 ± 4	95 ± 2	94 ± 2	97 ± 1
10-1670	Etretinate, free acid		79 ± 2	57 ± 2	81 ± 2	49 ± 3
13-7652	13- <i>cis</i> -Etretinate		91 ± 5	101 ± 1	96 ± 7	96 ± 4
12-7310	Etretinate metabolite		96 ± 7	59 ± 3	81 ± 1	57 ± 2
13-7410	Arotinoid, free acid		87 ± 4	66 ± 5	82 ± 1	61 ± 4
15-0778	Arotinoid, lacking a polar end group		90 ± 4	63 ± 8	91 ± 1	82 ± 2
15-1570	Arotinoid ethylsulphone		92 ± 2	74 ± 9	94 ± 3	86 ± 1
8-8717	Benzene-substituted all- <i>trans</i> -RA		97 ± 2	102 ± 6	99 ± 2	92 ± 4
	Retinol		114 ± 7	98 ± 7	99 ± 1	97 ± 2

duplicate and the results are expressed as the means $\pm$ S.E.M. of three experiments. Intra-assay and interassay coefficients of variation in the enzyme assay were $\pm 1\%$ and $\pm 5\%$ respectively.

RESULTS

Antagonism by RA of [¹²⁵I]T₄ binding to erythrocyte membranes

Studies conducted to define the binding characteristics of [¹²⁵I]T₄ and [¹²⁵I]T₃ revealed that two sites were present on the human erythrocyte membrane for both radiolabelled iodothyronines (Fig. 1). The K_d values for T₄ were 2.7×10^{-10} and 1.0×10^{-8} M for the high- and low-affinity sites respectively, and the maximal binding capacities were 76 fmol and 1.98 pmol/mg of protein. The K_d values for T₃ were 4.6×10^{-10} and 1.0×10^{-8} M, and the B_{max} values were 160 fmol and 2.5 pmol/mg of membrane protein for high- and low-affinity sites respectively. The ability of RA to displace [¹²⁵I]T₄ and [¹²⁵I]T₃ was identical to the abilities of the respective unlabelled hormones (Fig. 1). For example, the concentrations of T₄ and RA which displaced 50% of [¹²⁵I]T₄ were 6×10^{-9} M and 4×10^{-9} M respectively.

In another set of experiments, the abilities of T₄ and RA to displace [¹²⁵I]T₄ were compared with those of other retinoids. As shown in Fig. 2, RA (Ro 1-5488) and T₄ displaced [¹²⁵I]T₄

binding from erythrocyte membranes equally. When the RA ring is fully aromatized (Ro 8-8717), the molecule is completely inactive as a displacer of [¹²⁵I]T₄, as shown in Fig. 2 and summarized with other analogues in Table 1. An intact ring structure is apparently essential for iodothyronine binding displacement, since polyprenoic acid (E-5166) failed to influence [¹²⁵I]T₄ binding (Table 1). Similarly, when the polar end group of the side chain is in the *cis* configuration (Ro 4-3780), RA is inactivated. Studies with preparations of RA and 13-*cis*-RA from two different sources were comparable. Retinol failed to influence iodothyronine binding.

The addition of one methoxy and three methyl groups to the ring of the benzene-substituted molecule (Ro 10-1670) rendered the molecule nearly fully active as a displacer of [¹²⁵I]T₄ from its membrane binding sites. When a hydroxy group was substituted for the methoxy group (Ro 12-7310), the resultant compound retained potent displacing activity equal to that of RA (Table 1). Independent confirmation that the *trans* configuration of the polar end group is essential for activity was obtained from the finding that 13-*cis*-etretinate (Ro 13-7652) was completely inactive. Of the arotinoids tested, Ro 13-7410 was the most active in displacing T₄ from membranes. As depicted in Table 1, its structure bears similarity to that of all-*trans*-RA in that the

Fig 3. Effects of RA (Ro 1-5488) and seven retinoid analogues on T_4 -stimulable Ca^{2+} -ATPase activity in human erythrocyte membranes

The retinoid concentrations indicated on the abscissa were added to the incubation mixture as described in the Materials and methods section. Each data point represents the mean $\pm$ S.E.M. from three experiments performed in duplicate. Results are expressed as percentages of control T_4 stimulation ($0.181 \pm 0.029 \mu\text{mol}/90 \text{ min per mg}$) of enzyme activity in the presence of retinoid diluent (1% ethanol). C, control.

carboxylic group on the terminal phenyl ring is in an analogous configuration. This arrangement yields a molecule which is 85% as potent a displacer of $[^{125}\text{I}]\text{T}_4$ binding as all-*trans*-RA at 10^{-8} M . The substitution of the terminal carboxylic acid with an ethylsulphone group (Ro 15-1570) or its deletion (Ro 15-0778) rendered the resultant molecules considerably less potent (30% and 39% less active than all-*trans*-RA at 10^{-8} M respectively). Studies of displacement of $[^{125}\text{I}]\text{T}_3$ from red cell membranes by the above-named retinoid analogues yielded results comparable with those with T_4 .

We attempted to demonstrate specific $[^3\text{H}]\text{RA}$ binding to erythrocyte membranes. Radiolabelled ligand ($2 \times 10^{-9} \text{ M}$) was incubated with membrane aliquots at 4, 20 and 37°C without or with excess unlabelled RA or T_4 , for between 5 min and 2 h. While up to 23% of the tracer was bound, the addition of 10^{-6} M unlabelled RA or T_4 failed to influence $[^3\text{H}]\text{RA}$ binding.

Antagonism by RA of T_4 stimulation of Ca^{2+} -ATPase activity

RA blocks partially the thyroid hormone activation of Ca^{2+} -ATPase in the human erythrocyte membrane [15]. To determine the stereospecificity of this RA effect, red cell membranes were exposed to increasing concentrations of all-*trans*-RA or related compounds (10^{-10} M to 10^{-6} M). As Fig. 3 demonstrates, of the analogues tested, all-*trans*-RA (Ro 1-5488) inhibited T_4 -stimulable enzyme activity most potently. The effect was significant at a concentration of 10^{-9} M ($P < 0.001$; paired *t* test), and at 10^{-6} M the retinoid had inhibited T_4 stimulation by 88%. Ro 10-1670, the compound with the fully aromatized ring containing a methoxy group and three additional methyl groups, was nearly as active as all-*trans*-RA (Table 1). Substituting a hydroxy group for the methoxy group (Ro 12-7310) did not substantially alter the activity; Ca^{2+} -ATPase activity was $96 \pm 7\%$ of control at 10^{-10} M , $85 \pm 8\%$ at 10^{-9} M , $59 \pm 3\%$ at 10^{-8} M and $58 \pm 12\%$ at 10^{-6} M . The three arotinoids tested

(Ro 13-7410, Ro 15-0778 and Ro 15-1570), all of which had intermediate $[^{125}\text{I}]\text{T}_4$ displacing activities, demonstrated significant inhibition of the activation of Ca^{2+} -ATPase by T_4 . At 10^{-8} M , they were respectively 63%, 69% and 48% as potent as all-*trans*-RA. As was the case in the $[^{125}\text{I}]\text{T}_4$ binding studies, 13-*cis*-RA (Ro 4-3780), 13-*cis*-etretinate (Ro 13-7652), retinol and benzene-substituted all-*trans*-RA (Ro 8-8717) were completely devoid of activity in blocking the T_4 stimulation of Ca^{2+} -ATPase. Polyphenolic acid (E-5166) was also completely inactive (Table 1).

To determine whether these retinoid effects on T_4 stimulation of Ca^{2+} -ATPase were related only to the fatty acid side chain, sorbic and tiglic acids (C_6 and branched C_5 respectively) were tested and found to be inactive at concentrations up to 10^{-6} M . Moreover, neither influenced $[^{125}\text{I}]\text{T}_4$ binding (results not shown). Retinoid analogues were tested for their ability to inhibit T_3 stimulation of Ca^{2+} -ATPase activity; the findings were all very similar to those with T_4 .

The rate at which RA inhibits T_4 -stimulable Ca^{2+} -ATPase activity was determined. The effect of RA on Ca^{2+} -ATPase stimulation by T_4 was measured during assay periods of 30, 60 and 90 min. At these times the T_4 effect in the presence of RA (10^{-8} M) was 74%, 65% and 39% respectively of that found in the absence of the retinoid.

DISCUSSION

RA displaces $[^{125}\text{I}]\text{T}_4$ and $[^{125}\text{I}]\text{T}_3$ from their binding sites on human erythrocyte membranes. These sites, similar to those reported by another laboratory [10], are composed of both high- and low-affinity components (Fig. 1). That RA was equipotent with the unlabelled ligand as a specific binding displacer suggests that the interaction of this active retinoid with the membrane is also of high affinity and is recognized by the binding sites for T_4 . These findings are unexpected, because of the apparent lack of structural similarity between RA and T_4 .

The nature of the interaction between RA and the thyroid hormone binding site is, however, as yet obscure. The retinoid could be competing for the same site(s), or might be binding to a distinct site which, when occupied, causes an allosteric modification of the iodothyronine binding site. The data thus far obtained do not allow us to distinguish between these two possibilities. RA may be modifying the hormone binding site through a non-specific alteration in the adjacent lipid bilayer; however, this seems less likely because some of the other closely related retinoid analogues (retinol, 13-*cis*-RA, 13-*cis*-etretinate, polyphenolic acid, benzene-substituted RA), despite also being quite lipophilic, failed to influence hormone binding or activation of Ca^{2+} -ATPase even at concentrations which were 3.5 orders of magnitude greater than the apparent threshold for the RA effect ($5 \times 10^{-10} \text{ M}$).

We have shown previously that RA can inhibit non- T_4 -dependent Ca^{2+} -ATPase activity and that the enzyme activity is restored partially by the addition of exogenous calmodulin [17]. Moreover, $[^{125}\text{I}]\text{calmodulin}$ binding to human erythrocyte membranes was displaced by RA [17]. These effects are not seen with retinol or 13-*cis*-RA. Moreover, the rank order of retinoid analogues with respect to antagonism of calmodulin binding and action is similar to that for the blockade of thyroid hormone activation of Ca^{2+} -ATPase [18]. While a role for RA-induced antagonism of calmodulin action cannot be excluded in the present studies, the antagonism of the T_4 -stimulable Ca^{2+} -ATPase activity by RA reported here is likely related to the displacement of T_4 binding.

There appears to be generally good agreement between the stereochemical requirements for the blockade of enzyme activation by T_4 and for the displacement of $[^{125}\text{I}]\text{T}_4$ from its

membrane binding site. Both the RA ring structure and the fatty acid side chain are required for full activity. All-*trans*-RA was the most potent retinoid tested. Only Ro 15-0778 appeared to have greater potency in terms of blocking Ca^{2+} -ATPase activation by T_4 compared with inhibition of T_4 binding, but this analogue's activity was intermediate with respect to both actions.

The structure-activity relationships we describe here differ substantially from the limited number of reports already published utilizing multiple analogues of RA; however, the current studies probably represent actions mediated through receptor sites different from those which mediate the previously observed retinoid actions. The 13-*cis* configuration of the polar end group fails to influence RA activity substantially in a murine embryonal carcinoma cell culture system [19]. In that model the arotinoid containing the polar carboxylic acid group (Ro 13-7410) was also a potent inducer of differentiation. However, the removal of the end group or substitution of an ethylsulphone group for the carboxyl rendered the molecule inactive [19], unlike the results reported here.

Our results showing the lack of activity of the benzene-substituted all-*trans*-RA (Ro 8-8717) and the restoration of activity with the addition of one methoxy and three methyl groups to the ring structure (Ro 10-1670) are completely consistent with an early report of Chytil & Ong [20]. They found that these modifications respectively inactivated and restored binding activity of the molecules to cellular RA binding protein from rat testis, mouse papilloma and human breast tumours [20].

While all-*trans*-RA is a potent displacer of thyroid hormone binding, we were unable to demonstrate any displacement of [^3H]RA by either unlabelled all-*trans*-RA or T_4 . This is not surprising, considering the hydrophobic nature of retinoids. Similar difficulties were encountered in demonstrating steroid hormone binding to plasma membranes, despite the well-documented action of these compounds at the membrane [21]. Demonstration of a specific RA binding site may await the isolation of a functional membrane receptor protein extracted from the bilayer.

The present findings raise the possibility that RA may be an endogenous modulator of thyroid hormone action. The alteration in the dose-response curves for T_4 and T_3 by RA [15] and the displacement of the binding of these ligands to red cell membranes occurred at physiologically relevant RA concentrations [22]. The two-order shift for maximal activation by T_4 and T_3 caused by $5 \times 10^{-9} \text{ M}$ -RA [15] suggests that the effective hormone concentrations found under normal conditions *in vivo* may not be faithfully reproduced under conditions *in vitro* if physiological concentrations of RA are not present. Such findings underscore

the complexities associated with the regulation of cellular metabolism and the caution needed in the interpretation of data generated in artificial models.

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