Sex-dependent inhibition by retinoic acid of thyroid-hormone action on rabbit reticulocyte Ca^{2+} -ATPase activity

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The interaction was examined *in vitro* of retinoic acid and thyroid hormone with rabbit reticulocyte Ca^{2+} -ATPase. L-Thyroxine (T_4) (0.1 nM) stimulated female-source Ca^{2+} -ATPase activity (+21 %; $P < 0.03$) and inhibited male-source enzyme (–20 %; $P < 0.05$). Addition of retinoic acid (10 nM–1 μM) did not influence T_4 -inhibitable male-source Ca^{2+} -ATPase, but caused a 52 % loss of T_4 effect on the female-source enzyme. Incubation of female-source membranes with testosterone caused the enzyme response to T_4 and retinoic acid to become that of male-source membranes, and the male-source enzyme response was converted into the ‘female’ pattern by exposure to 17β -oestradiol. We postulate that a membrane-associated sex-steroid-dependent factor imparts a gender-specific interaction of thyroid hormone and retinoic acid on Ca^{2+} -ATPase, and that ultimately the factor is shed during erythrocyte maturation.

INTRODUCTION

Plasma-membrane Ca^{2+} -stimulatable Mg^{2+} -dependent ATPase (Ca^{2+} -ATPase, EC 3.6.1.3) activity is principally regulated by intracellular Ca^{2+} concentration, through the calmodulin- Ca^{2+} complex [1]. Ca^{2+} -ATPase activity in human and rabbit mature erythrocyte membranes is also stimulated *in vitro* by physiological concentrations (0.1 nM) of L-thyroxine (T_4) and 3,3',5-L-tri-iodothyronine (T_3) [2–4]; stimulation of human erythrocyte Ca^{2+} -ATPase by endogenous thyroid hormone also occurs *in vivo* [5]. In contrast with observations made in mature erythrocyte membranes, rabbit reticulocyte membrane Ca^{2+} -ATPase activity responds to thyroid hormone on a sex-specific basis: female-source reticulocyte membranes have Ca^{2+} -ATPase activity that is stimulated *in vitro* by thyroid hormone, whereas male-source enzyme is inhibited [6]. This differential response is a function of ambient sex-steroid environment of the cell membranes *in vivo* and *in vitro*, rather than a genetic sex-determined feature of Ca^{2+} -ATPase. Enzyme stimulation by T_4 in female-source reticulocyte membranes can be converted into thyroid-hormone-induced enzyme inhibition by incubating either membranes or whole reticulocytes with physiological concentrations of testosterone [6,7].

We have recently shown that retinoic acid, but not retinol, partially blocks thyroid-hormone activation *in vitro* of human mature erythrocyte membrane Ca^{2+} -ATPase activity [8]. The retinoic acid effect in this system demonstrates competitive inhibition of thyroid-hormone action on the enzyme. We have also determined that retinoic acid displaces [^{125}I] T_4 and [^{125}I] T_3 from human erythrocyte membrane binding sites [8].

In the present study we have examined the action of retinoic acid on the sex-specific response of rabbit reticulocyte Ca^{2+} -ATPase to iodothyronines, in order to establish whether the interaction of thyroid hormone and retinoic acid in this model is conditioned by the sex-steroid milieu at the membrane enzyme site.

MATERIALS AND METHODS

Hormones and reagents

T_4 , all-*trans*-retinoic acid, retinol, testosterone, 17β -oestradiol and Na_2ATP were obtained from Sigma, and [^{125}I] T_4 (1250 $\mu\text{Ci}/\mu\text{g}$) was from DuPont–New England Nuclear.

Induction of reticulocytosis and preparation of reticulocyte membranes

Reticulocytosis was induced in adult male and female rabbits by repeated venipuncture and removal of 40–45 ml of ear arterial blood, in accordance with our previously published schedule [6]. Reticulocyte membranes from 40 ml of heparinized blood containing 35–55 % reticulocytes were prepared by hypo-osmotic lysis [2]. Membranes were washed twice in 0.9 % NaCl and twice in 10 mM-Tris/HCl, pH 7.45, and stored in that buffer at -70°C until used within 72 h.

Ca^{2+} -ATPase activity

Enzyme activity was measured by our previously published method [2]. Activity was defined as the difference in ATP hydrolysis in the presence and absence of 20 μM free Ca^{2+} , with liberated P_i quantified by the Malachite Green method [9], as μmol of P_i/mg of membrane protein per 30 min assay period. Membrane protein was quantified by the method of Lowry *et al.* [10], with BSA as standard. Experimental results are reported as means $\pm$ S.E.M. of three independent experiments in each of which the results of duplicate samples were averaged ($n = 3$). Statistical significance was measured by one-way analysis of variance (ANOVA).

Effect of hormones or retinoids on membrane enzyme activity

Reticulocyte membranes were incubated at 37°C with retinoic acid, retinol, T_4 , testosterone and/or 17β -oestradiol for 60 min before enzyme assay. Diluents were 1 % ethanol for retinoids, testosterone and 17β -oestradiol, or 10 mM-Tris/HCl, pH 7.45,

Abbreviations used: Ca^{2+} -ATPase, Ca^{2+} -stimulatable Mg^{2+} -dependent ATPase; T_4 , L-thyroxine; T_3 , 3,3',5-L-tri-iodothyronine.

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for T_4 ; control samples of membranes contained diluent, but no hormone or retinoid. Ethanol (1%) had no effect itself on Ca^{2+} -ATPase activity.

$[^{125}I]T_4$ -binding studies

Reticulocyte membranes (0.5 mg/tube) were incubated for 60 min at 37 °C with $[^{125}I]T_4$ (80 pM) in 10 mM-Tris/HCl, pH 7.45, and unlabelled T_4 , retinoic acid, testosterone (5 nM) or 17 β -oestradiol (5 nM), as described previously [8]. Separation of bound from unbound ligand was by centrifugation at 16000 g for 10 min, followed by one wash of the pellet. All experiments were conducted in duplicate, and results presented are means $\pm$ S.E.M. of two experiments ($n = 4$). The diluent, 1% ethanol, had no effect on thyroid-hormone binding to reticulocyte membranes.

RESULTS

Reticulocyte-enriched membrane basal Ca^{2+} -ATPase activity (without thyroid-hormone addition) was comparable in male- and female-source membranes: 1.02 ± 0.06 and 1.01 ± 0.02 μ mol of P_i /mg per 30 min respectively. T_4 (0.1 nM) stimulated female-source membrane Ca^{2+} -ATPase activity (+21%, $P < 0.03$) and significantly inhibited male-source enzyme activity (−20%, $P < 0.05$).

In female-source reticulocyte-enriched membranes, T_4 stimulation of Ca^{2+} -ATPase activity was inhibited by retinoic acid (10 nM–1 μ M) in a dose-dependent manner ($P < 0.003$) (Fig. 1). The retinoic acid effect in these membranes was comparable with that obtained in human mature erythrocyte membranes obtained from either gender [8]. In contrast, retinoic acid (10 nM–1 μ M) did not affect the inhibition of T_4 of Ca^{2+} -ATPase activity in male-source reticulocyte-enriched membranes (Fig. 1). Retinol had no effect on male- or female-source reticulocyte Ca^{2+} -ATPase, in either the presence or the absence of T_4 (results not shown).

Stimulation of female-source reticulocyte membrane enzyme activity by T_4 , and the blockade of that effect by retinoic acid, are shown in Fig. 2(a). When female-source reticulocyte membranes were preincubated with testosterone (5 nM) before enzyme assay, T_4 inhibited Ca^{2+} -ATPase activity (Fig. 2b), a response typical of male-source reticulocyte membranes (Fig. 2c). Addition of retinoic acid to testosterone-treated female-source membranes failed to influence the enzyme inhibition by T_4 (Fig. 2b), similar to the results observed in male-source reticulocytes (Fig. 2c). Conversely, enzyme inhibition by T_4 in male-source reticulocyte membranes was converted into the female pattern of response (enzyme stimulation) by preincubation of membranes with 17 β -oestradiol (5 nM); this stimulation was inhibited by retinoic acid (Fig. 2d).

In the absence of exogenous T_4 , retinoic acid (10 nM–10 μ M) decreased membrane Ca^{2+} -ATPase activity equally in female- and male-source reticulocyte membranes; this effect was concentration-dependent and not altered by either 17 β -oestradiol or testosterone (results not shown). Mature erythrocyte membrane Ca^{2+} -ATPase activity from male and female rabbits responded to T_4 and retinoic acid in a manner similar to that seen in human mature erythrocyte membranes [8], in that thyroid-hormone stimulation was inhibited by retinoic acid, and was unaffected by testosterone and 17 β -oestradiol (results not shown).

The effects of retinoic acid, T_4 and sex steroids on labelled- T_4 binding to reticulocyte membranes are shown in Table 1. Non-radiolabelled T_4 and retinoic acid displaced tracer to a similar extent from both male- and female-source reticulocyte membranes. Non-specific binding of T_4 , at a total T_4 concentration of 1 μ M, was 46% of the binding with tracer alone. Retinoic acid (1 μ M) displaced 88% of specific T_4 binding. The preincubation

Fig. 1. Interaction of T_4 and retinoic acid on reticulocyte membrane Ca^{2+} -ATPase activity from female and male rabbits

Enzyme activities without hormone or retinoid were 1.02 ± 0.06 and 1.01 ± 0.02 μ mol of P_i /mg per 30 min in male- and female-source membranes respectively. The change in enzyme activity with the addition of T_4 (0.1 nM) is shown on the ordinate. The control values (C) demonstrate enzyme stimulation by T_4 in female-source membranes, and inhibition by T_4 in male-source membranes. Retinoic acid (10 nM and 1 μ M) inhibited the T_4 -induced enhancement of Ca^{2+} -ATPase in female-source membranes in a dose-dependent manner, but did not influence the inhibitory effect of T_4 in male-source membranes.

Fig. 2. Interaction of T_4 , retinoic acid and sex hormones on rabbit reticulocyte membrane Ca^{2+} -ATPase activity

Control enzyme activities were as given for Fig. 1. The ordinate shows the change in enzyme activity with addition of T_4 (0.1 nM). The female-source reticulocyte enzyme is stimulated by T_4 (a, ● within square); this stimulation is decreased by retinoic acid (10 nM and 1 μ M). Female-source reticulocyte membranes exposed to testosterone (5 nM) show enzyme inhibition by T_4 (b, ○ within square), which is unaffected by retinoic acid. A similar response is seen with male-source reticulocyte membranes (c). Male-source membranes preincubated with 17 β -oestradiol (5 nM) (d) display responses seen with female-source membranes.

Table 1. Effect of T_4 , retinoic acid, testosterone and 17β -oestradiol on binding of radiolabelled T_4 to male- female-source rabbit reticulocyte membranes

Values are expressed as means $\pm$ S.E.M. of two experiments, each of which was performed with duplicate samples ($n = 4$): ^a $P < 0.001$ by ANOVA, comparing binding to a range of unlabelled- T_4 concentrations with binding of tracer alone; ^b $P < 0.001$ by ANOVA, comparing binding of tracer to a range of retinoic acid concentrations with binding of tracer T_4 alone.

		Percentage of [125 I] T_4 bound			
		Male		Female	
		Without oestradiol	Oestradiol (5 nM)	Without testosterone	Testosterone (5 nM)
[125 I] T_4	80 pM	34.6 $\pm$ 0.6	33.7 $\pm$ 0.5	35.8 $\pm$ 0.7	33.5 $\pm$ 0.2
+ T_4	10 nM	29.6 $\pm$ 0.9	29.5 $\pm$ 1.0	29.2 $\pm$ 0.9	28.3 $\pm$ 0.8
	1 μ M	16.0 $\pm$ 0.3 ^a	16.6 $\pm$ 1.0 ^a	16.2 $\pm$ 1.0 ^a	16.7 $\pm$ 0.9 ^a
+ Retinoic acid	10 nM	29.1 $\pm$ 0.6	29.8 $\pm$ 0.6	29.7 $\pm$ 0.4	28.9 $\pm$ 0.4
	1 μ M	18.2 $\pm$ 1.3 ^b	17.1 $\pm$ 0.6 ^b	16.7 $\pm$ 0.5 ^b	16.6 $\pm$ 0.6 ^b

of 17β -oestradiol (5 nM) with male-source membranes, or of testosterone (5 nM) with female-source membranes, for up to 60 min before binding assay, failed to alter the binding of tracer T_4 or its displacement of unlabelled T_4 or retinoic acid.

DISCUSSION

Rabbit reticulocyte membranes, but not those from mature erythrocytes, contain Ca^{2+} -ATPase activity which manifests donor sex-specific response to thyroid hormone *in vitro* [6,7]. This response of reticulocyte membranes reflects the ambient concentration of oestrogen or androgen in the membrane milieu, and is not a trait conditioned in the erythroid cell line by gender of the cell donor [6,7]. We have previously shown that the thyroid-hormone response of Ca^{2+} -ATPase can be altered by exposing intact reticulocytes to specific gonadal steroids before membrane preparation, or by exposing reticulocyte membranes to the steroids [6]. It is therefore evident that sex-steroid conditioning of the cell membranes is not lost during cell lysis and membrane preparation, including several washes, perhaps owing to solubilization of the hormones in the membrane lipid bilayer.

We have shown previously in the mature human erythrocyte that retinoic acid and thyroid hormone (either T_3 or T_4) interact at the plasma-membrane Ca^{2+} -ATPase, and have suggested that inhibition by retinoic acid of thyroid-hormone-stimulatable Ca^{2+} -ATPase is related to displacement by the retinoid of thyroid hormone from membrane binding sites [8]. The mature erythrocyte membrane in mammals is substantially different from that of reticulocytes, in terms of physical and biochemical characteristics [11–13]. Yet it is evident that T_4 and retinoic acid interact in the female-source reticulocyte in a manner similar to that seen in mature erythrocyte membranes. In the male-source reticulocyte membrane, T_4 inhibits Ca^{2+} -ATPase activity, an effect which is not blocked by retinoic acid; this suggests that retinoic acid does not completely inhibit T_4 binding to the membrane, or that in androgen-conditioned membranes the retinoid does not have access to the site of interaction of T_4 with the membrane, or that in androgen-conditioned membranes the retinoid does not have access to the site of interaction of T_4 with the membrane, at or near the Ca^{2+} -ATPase. With the addition to male-source membranes of oestrogen, however, T_4 stimulation of the enzyme, and the sensitivity of that effect to retinoic acid, are again apparent.

We speculate that an androgen-dependent factor exists in rabbit reticulocyte membranes which inhibits the action of

thyroid hormone and its interaction with retinoic acid on the Ca^{2+} -ATPase, and which is inactivated in the presence of oestrogen. Loss of the inhibitor during erythroid cell maturation would result in the similar responses of male- and female-source mature erythrocyte membrane Ca^{2+} -ATPase to thyroid hormone and retinoic acid that we have seen, and is consistent with the evidence of others that during maturation of the reticulocyte a number of transport enzymes and receptors are shed [14–16]. An alternative explanation would be that an oestrogen-activated reticulocyte factor which permits thyroid hormone to stimulate Ca^{2+} -ATPase, and which can be blocked by androgen in the reticulocyte membrane, becomes constitutive in mature erythrocytes of both sexes.

We have demonstrated no effect of 17β -oestradiol or testosterone on the binding of radiolabelled T_4 to male- or female-source reticulocyte membranes. We therefore conclude that these gender-specific actions of thyroid hormone and retinoic acid occur distal to the association of the ligands with their binding sites on reticulocyte membranes, and involve the interaction of a reticulocyte- and sex-steroid-specific factor which resides within the lipid bilayer of the plasma membrane.

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