Structure–activity relationships of retinoids as inhibitors of calmodulin-dependent human erythrocyte Ca^{2+} -ATPase activity and calmodulin binding to membranes

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All-*trans* retinoic acid displaces the binding of radiolabelled calmodulin to human erythrocyte membranes, and inhibits the activity of plasma membrane Ca^{2+} -stimulated, Mg^{2+} -dependent ATPase (Ca^{2+} -ATPase; EC 3.6.1.3). This enzyme is dependent upon the action of calmodulin. In this study we explored the structural attributes of the retinoids which confer this ability to inhibit enzyme activity and calmodulin binding. With respect to the fatty acid side-chain, a clear requirement for inhibition is a *trans*-configuration of the polar end-group. The importance of the ring structure is indicated by the ineffectiveness of polyprenoic acid and a benzene ring retinoid analogue as inhibitors of enzyme activity and calmodulin binding. There was good correlation between the relative potencies of the analogues as enzyme inhibitors and as inhibitors of calmodulin binding. The ability of selected retinoid analogues, at physiological concentrations with respect to all-*trans* retinoic acid, to inhibit erythrocyte Ca^{2+} -ATPase activity and membrane binding of calmodulin underscores the structurally specific effects of these compounds on the interaction of calmodulin with the membrane-bound enzyme.

INTRODUCTION

The activity of erythrocyte membrane Ca^{2+} -stimulated Mg^{2+} -dependent ATPase (Ca^{2+} -ATPase; EC 3.6.1.3) is dependent upon the presence of the ubiquitous intracellular Ca^{2+} -binding protein calmodulin [1]. The activity of this enzyme is inhibited by a variety of calmodulin antagonists, which are thought to interact with calmodulin at one or more specific binding sites [2–5], and prevent its binding to those sites. Among these calmodulin inhibitors are *N*-(6-aminohexyl)-5-chloronaphthalene-1-sulphonamide (W-7) [6], calmidazolium [7], phenothiazines such as trifluoperazine [8], Ca^{2+} channel blockers [9,10], and the anti-arrhythmic agent amiodarone [11]. Several fatty acids, including oleic acid [12], also inhibit human erythrocyte membrane Ca^{2+} -ATPase activity. The calmodulin antagonism by fatty acids apparently does not depend upon direct interaction of fatty acids with the Ca^{2+} -binding protein, but rather upon their interaction with the plasma membrane at or near the calmodulin-binding site [12].

We have recently reported the inhibition of erythrocyte membrane Ca^{2+} -ATPase activity by physiological concentrations of all-*trans* retinoic acid [13,14]. In contrast, retinol, retinal, 13-*cis* retinoic acid, and a benzene-ring-substituted all-*trans* retinoic acid all failed to inhibit the enzyme [14]. All-*trans* retinoic acid has also been shown to displace radiolabelled calmodulin from erythrocyte membrane binding sites [14], suggesting a co-ordinate effect of retinoid analogues on both membrane binding of calmodulin and calmodulin-stimulatable Ca^{2+} -ATPase activity. We have therefore studied a number of retinoids in the Ca^{2+} -ATPase and calmodulin-binding assays in order to define the contributions of both the ring structure and the side-chain components of the retinoids to the effects that we have observed.

MATERIALS AND METHODS

Reagents

Bovine brain calmodulin, all-*trans* retinoic acid, retinol, retinal,

13-*cis* retinoic, and sorbic and tiglic acids were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Additional retinoids, as well as all-*trans* retinoic acid and 13-*cis* retinoic acid, were kindly provided by Dr. Stanley Shapiro (Hoffmann–La Roche, Nutley, NJ, U.S.A.). ^{125}I -Calmodulin was obtained from New England Nuclear (Boston, MA, U.S.A.), and had a specific radioactivity of 90.8 $\mu\text{Ci}/\text{mg}$.

Erythrocyte membranes

Human erythrocyte membranes were prepared from fresh heparinized blood obtained from healthy donors with written informed consent, as described [15]. After washing in 10 mM-Tris/HCl buffer, pH 7.45, membranes were stored in the same buffer at -70°C until used for enzyme assay or calmodulin-binding studies within 10 days.

Ca^{2+} -ATPase assay

Erythrocyte membrane Ca^{2+} -ATPase activity was measured as the difference in P_i liberated from ATP in the presence and absence of 20 μM free Ca^{2+} , and was expressed in μmol of $\text{P}_i/90$ min per mg of membrane protein. P_i was measured by the method of Lanzetta *et al.* [16] and protein was measured by the method of Lowry *et al.* [17]. We have demonstrated that enzyme activity is linear for 90 min after addition of 1 mM- Na_2ATP , in the presence or absence of retinoic acid. Enzyme activity with retinoic acid (10^{-8} M) was 68%, 61% and 55% of that without the retinoid at 30, 60 and 90 min of the assay respectively. Assays were carried out in duplicate, and results are expressed as the means $\pm$ S.E.M. of three or more studies. Retinoids were dissolved in 95% ethanol, stored at -20°C in the dark, and diluted in 10 mM-Tris/HCl, pH 7.45, to the working concentrations indicated, in 1% ethanol. The membranes were preincubated with a retinoid and/or calmodulin, or appropriate diluent controls, for 60 min at 37°C before addition of Na_2ATP . The retinoic acid effect, however, was evident after the 90 min assay time in the absence of preincubation.

Abbreviations used: Ca^{2+} -ATPase, Ca^{2+} -stimulated Mg^{2+} -dependent ATPase; W-7, *N*-(6-aminohexyl)-5-chloronaphthalene-1-sulphonamide.

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Table 1. Structure-activity relationships of retinoid analogues

Ro No.	Name	Basic chemical structure	Concn. ...	Ca ²⁺ -ATPase activity (% of control)		Specific calmodulin binding to erythrocyte membranes (% of control)	
				10 ⁻¹⁰ M	10 ⁻⁸ M	10 ⁻¹⁰ M	10 ⁻⁸ M
1-5488	All- <i>trans</i> -retinoic acid (tretinoin)			82 ± 4	55 ± 2	76 ± 7	43 ± 1
4-3780	13- <i>cis</i> -Retinoic acid			103 ± 5	105 ± 5	88 ± 4	86 ± 2
E-5166	Polyprenoic acid			101 ± 1	102 ± 1	98 ± 4	100 ± 4
8-8717	Benzene-substituted all- <i>trans</i> -retinoic acid			100 ± 1	98 ± 1	98 ± 2	99 ± 1
10-1670	Etretinate, free acid			81 ± 3	55 ± 3	75 ± 1	54 ± 2
13-7652	13- <i>cis</i> -Etretinate			102 ± 1	102 ± 2	100 ± 3	101 ± 2
17-7310	Etretinate metabolite			93 ± 4	69 ± 1	75 ± 3	57 ± 1
13-7410	Arotinoid, free acid			80 ± 8	55 ± 1	77 ± 3	52 ± 3

¹²⁵I-Calmodulin-binding studies

Erythrocyte membranes (1.0 mg/ml), a retinoid or diluent, and ¹²⁵I-calmodulin (6 × 10⁻¹² M) were incubated at 37 °C for 60 min in 10 mM-Tris, pH 7.45. Separation of bound and free calmodulin was accomplished by centrifugation as previously described [14]. Without added retinoid, there was 9.5 % binding of tracer calmodulin, 63 % of which was non-displaceable, as defined by the displacement of tracer by 10⁻⁶ M unlabelled calmodulin. Studies were carried out in duplicate, and the results presented are from three or more experiments.

RESULTS

Ca²⁺-ATPase activity

Control erythrocyte Ca²⁺-ATPase activity without exogenous calmodulin or retinoid analogue was 0.543 ± 0.023 μmol of P_i/90 min per mg of protein. Retinoic acid (Ro 1-5488) caused 18 and 45 % inhibition of enzyme activity respectively at concentrations of 10⁻¹⁰ and 10⁻⁸ M (Table 1). In contrast, 13-*cis* retinoic acid (Ro 4-3780) had no significant inhibitory effect, as we have previously reported [14]. Polyprenoic acid (E-5166), an open-ring analogue of retinoic acid, and benzene-ring-substituted all-*trans* retinoic acid (Ro 8-8717) were similarly ineffective. Whereas etretinate (Ro 10-1670), an analogue with a benzene ring modified by the addition of a carboxymethyl and three methyl groups, was inhibitory to the same degree as all-*trans* retinoic acid, 13-*cis* etretinate (Ro 13-7652) was inactive. A metabolite of etretinate, Ro 12-7310, with a hydroxyl group in place of the carboxymethyl group, was almost as potent an

inhibitor as was etretinate. The free acid of arotinoid (Ro 13-7410), which contains a fully saturated ring and two unsaturated rings, also inhibited enzyme activity to the same degree as all-*trans* retinoic acid. Arotinoid lacking the terminal carboxyl group (Ro 15-0778), and an arotinoid analogue with an ethylsulphone group in place of the terminal carboxyl group (Ro 15-1570), inhibited enzyme activity by 27 % and 20 % respectively (i.e. 60 % and 44 % as active as all-*trans* retinoic acid) at a concentration of 10⁻⁸ M. We have previously reported that retinol and retinal do not inhibit Ca²⁺-ATPase activity [14]. Although some long-chain fatty acids, such as oleic (C_{18:1}) and stearic (C_{18:0}) acids inhibit Ca²⁺-ATPase activity [12], two short-chain fatty acids, sorbic (C_{6:2}) and tiglic (branched C_{5:1}) acids, were without effect on Ca²⁺-ATPase (results not shown).

We have previously reported that all-*trans* retinoic acid (10⁻⁸ M) shifted the calmodulin dose-response curve of Ca²⁺-ATPase activity to the right, with a decrease in V_{max}. [14]. We compared the calmodulin response in the presence of 13-*cis* retinoic acid, all-*trans* retinoic acid (both at 10⁻⁸ M) or diluent. As seen in Fig. 1, the 13-*cis* congener had no effect on calmodulin-stimulatable enzyme activity, whereas all-*trans* retinoic acid caused a decrease in V_{max} and an increase in K_m. Even at a concentration of 10⁻⁶ M, 13-*cis* retinoic acid failed to influence the calmodulin dose-response curve.

Membrane binding of ¹²⁵I-calmodulin

All-*trans* retinoic acid inhibited specific radiolabelled calmodulin binding to erythrocyte membranes by 24 and 57 % when present at 10⁻¹⁰ and 10⁻⁸ M respectively. The results of the

Fig. 1. Response of erythrocyte Ca^{2+} -ATPase activity to exogenous purified calmodulin in the presence of all-*trans* retinoic acid, 13-*cis* retinoic acid or diluent (control)

Results shown are means $\pm$ S.E.M. of three experiments performed in duplicate. Without retinoid, maximal enzyme stimulation by calmodulin is seen with a calmodulin concentration of 6×10^{-8} M. In the presence of all-*trans* retinoic acid (■, 10^{-8} M) there is a decrease in V_{max} , and a shift of the curve to the right. With 13-*cis* retinoic acid (○, 10^{-8} M) the dose-response curve is superimposed on that of the control samples (●).

effects of retinoid analogues on calmodulin binding are summarized in Table 1. 13-*cis* Etretinate (Ro 13-7652), the benzene ring analogue (Ro 8-8717) and polyprenonic acid (E-5166) did not inhibit calmodulin binding. Although 13-*cis* retinoic acid (Ro 4-3780) had a small inhibitory effect on calmodulin binding, this effect was not dose-dependent, and concentrations up to 10^{-4} M produced no further displacement. Calmodulin binding was inhibited by etretinate (Ro 10-1670), the etretinate metabolite (Ro 12-7310), and the free acid of arotinoid (Ro 13-7410) to an extent comparable with that of all-*trans* retinoic acid. Thus compounds which inhibited Ca^{2+} -ATPase activity also displaced radiolabelled calmodulin from membrane binding sites, whereas compounds which did not alter enzyme activity failed to alter calmodulin binding.

DISCUSSION

All-*trans* retinoic acid has been shown to have an anti-calmodulin effect at the erythrocyte plasma membrane, namely the inhibition of basal and exogenous calmodulin-stimulated Ca^{2+} -ATPase activity. This action appears to be mediated by displacement of calmodulin binding from erythrocyte membranes [14]. Unlike other calmodulin antagonists, such as Ca^{2+} channel blockers [18,19], W-7 [19,20] and amiodarone [11], which bind directly to specific sites on the calmodulin molecule, retinoic acid inhibits the binding of calmodulin to the membrane by interacting with the membrane at or close to the site where calmodulin binds to Ca^{2+} -ATPase. That there appears to be no direct interaction of retinoic acid with calmodulin is indicated by the lack of competition between retinoic acid and 8-anilino-naphthalene-1-sulphonic acid for binding sites on calmodulin that are involved in Ca^{2+} -ATPase stimulation [14], and by the fact that retinoic acid does not inhibit calmodulin-stimulated phosphodiesterase activity [14].

The retinoid effect on the membrane binding of calmodulin and Ca^{2+} -ATPase activity is dependent on the configuration of both the side-chain polar group and the ring structure. The 13-*trans* configuration is essential, as demonstrated by the lack of inhibition by 13-*cis* retinoic acid and 13-*cis* etretinate of enzyme activity and calmodulin binding. If the side-chain is linear, then

a terminal polar group of sufficient size, e.g. a carboxyl group, appears to be necessary; retinol and retinal are ineffective. With the presence of a benzene ring on the side-chain, as in the arotinoids, inhibition of enzyme activity and calmodulin binding may be seen in the absence of a terminal polar group. The ring structure of retinoic acid is also essential, as demonstrated by the lack of activity of polyprenonic acid and the benzene ring analogue. However, the addition of a carboxymethyl group and three methyl groups to the benzene ring structure, as in etretinate, restored anti-calmodulin activity. This is also true of the etretinate metabolite that has a hydroxyl group in place of the carboxymethyl group.

The anti-calmodulin effect of the retinoids that we have observed is similar to that caused by long-chain fatty acids [12]. In our prior study, we demonstrated that oleic ($\text{C}_{18:1}$), stearic ($\text{C}_{18:0}$) and myristic ($\text{C}_{14:0}$) acids, but not palmitic ($\text{C}_{16:0}$), palmitoleic ($\text{C}_{16:1}$), myristoleic ($\text{C}_{14:1}$) and lauric ($\text{C}_{12:0}$) acids, inhibited erythrocyte Ca^{2+} -ATPase activity. There was also inhibition by oleic acid of calmodulin binding to membranes [12]. We have examined the effect of sorbic ($\text{C}_{6:2}$) and tiglic ($\text{C}_{5:1}$, branched) acids on membrane Ca^{2+} -ATPase activity and found no effect at concentrations up to 10^{-6} M. This information, coupled with the ineffectiveness of polyprenonic acid, indicates that both a *trans*-configured side-chain and an intact ring structure are necessary for the anti-calmodulin effect reported here.

Retinoic acid also inhibits thyroid-hormone-stimulated Ca^{2+} -ATPase activity in human erythrocyte membranes. That effect appears to be, at least in part, the consequence of displacement by the retinoid of thyroid hormone from plasma membrane binding sites [13]. As thyroid hormone stimulation of this enzyme requires the presence of calmodulin [21], the anti-calmodulin effect of the retinoids could also play a role. However, the retinoid effect appears to be competitive with respect to thyroid hormone [13], whereas the addition of exogenous calmodulin does not reverse retinoid inhibition of the thyroid hormone effect. The ranking of inhibitory potency of analogues relative to the thyroid hormone effect is very similar to the ranking with respect to the anti-calmodulin action described above. It is therefore apparent that selected retinoids, with a *trans*-configured side-chain containing a polar end group and an intact ring structure, act within the plasma membrane to inhibit the effects of both calmodulin and thyroid hormone on Ca^{2+} -ATPase activity.

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