Retinoic Acid Inhibits Calmodulin Binding to Human Erythrocyte Membranes and Reduces Membrane Ca^{2+}-Adenosine Triphosphatase Activity

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Abstract

Ca^{2+}-ATPase activity in human red cell membranes is dependent on the presence of calmodulin. All *trans*-retinoic acid inhibited human red cell membrane Ca^{2+}-ATPase activity in vitro in a concentration-dependent manner (10^{-8} to 10^{-4} M). In contrast, retinol, retinal, 13-*cis*-retinoic acid and the benzene ring analogue of retinoic acid did not alter enzyme activity. Purified calmodulin (up to 500 ng/ml, 3×10^{-8} M) added to red cell membranes, in the presence of inhibitory concentrations of retinoic acid, only partially restored Ca^{2+}-ATPase activity. ^{125}I-Calmodulin bound to red cell membranes was displaced by unlabeled retinoic acid (50% reduction at 10^{-8} M retinoic acid), as effectively as by unlabeled calmodulin. Another calmodulin-stimulable enzyme, bovine brain cyclic nucleotide phosphodiesterase, was unaffected by retinoic acid. 8-Anilino-1-naphthalene sulfonic acid bound to calmodulin, studied spectrofluorometrically, was not displaced by retinoic acid. Thus, retinoic acid inhibits calmodulin binding to red cell membranes, reducing calmodulin-stimulable Ca^{2+}-ATPase activity. Retinoic acid does not directly interact with calmodulin, but rather exerts its effect by interfering with calmodulin access to the membrane enzyme. These effects occur at physiological concentrations of the retinoid. (*J. Clin. Invest.* 1990. 85:1999–2003.) retinoic acid • calmodulin • Ca^{2+}-ATPase

Introduction

Calmodulin is a ubiquitous intracellular Ca^{2+}-binding protein (1) among whose functions are regulation of the activities of plasma membrane Ca^{2+}-stimulated, Mg^{2+}-dependent adenosine triphosphatase (Ca^{2+}-ATPase[1]; EC 3.6.1.3) (2), cytoplasmic cyclic nucleotide phosphodiesterase (3) and certain protein kinases (4). Calmodulin possesses discrete domains including binding sites for Ca^{2+} (5) and several hydrophobic sites at which it interacts with target enzymes (6). Calmodulin antagonists are thought to bind to the hydrophobic site(s), although the interaction could occur elsewhere and allosterically affect enzyme activation by calmodulin.

Our laboratory has described the susceptibility of human red cell membrane Ca^{2+}-ATPase to stimulation in vitro by physiological concentrations of thyroid hormone (7, 8). This action of thyroid hormone and its analogues requires the presence of calmodulin (9), but the hormone and calmodulin do not interact directly in this system (10). Recently, we reported that retinoic acid blocks the activation by thyroid hormone of human erythrocyte Ca^{2+}-ATPase (11). This action has stringent stereochemical requirements; retinol, retinal and 13-*cis*-retinoic acid all failed to mimic all-*trans*-retinoic acid (12). The mechanism by which retinoic acid interferes with thyroid hormone action at the plasma membrane Ca^{2+}-ATPase depends upon competition between retinoic acid and L-thyroxine (T_4) or 3,5,3'-L-triiodothyronine (T_3) for hormone-binding sites on the red cell membrane (12). However, retinoic acid also inhibits Ca^{2+}-ATPase activity in the absence of thyroid hormone (11). In this report we show that retinoic acid interferes with the binding of calmodulin to the membrane enzyme, accounting for the inhibition of enzyme activity. This observation has implications for a variety of biological effects ascribed to retinoic acid.

Methods

Reagents. Bovine brain calmodulin, all *trans*-retinoic acid, retinol, retinal, 13-*cis*-retinoic acid, 8-anilino-1-naphthalene sulfonic acid (ANS), *N*-(6-aminohexyl)-5-chloro-1-naphthalene sulfonamide (W-7), bovine brain 3':5'-cyclic nucleotide phosphodiesterase and *Crotalus atrox* venom 5'-nucleotidase were obtained from Sigma Chemical Co. (St. Louis, MO). RO 8-8717, an all-*trans*-, benzene ring-substituted retinoic acid analogue was kindly provided by Dr. Stanley Shapiro of Hoffmann-La Roche (Nutley, NJ). ^{125}I-Calmodulin was purchased from New England Nuclear (Boston, MA), and had a specific activity of 90.8 μCi/μg.

Erythrocyte membranes. Human red cell ghosts were prepared hypotonically from heparinized blood, as previously described (7). The membranes were stored in 10 mM Tris, pH 7.45, at −70°C until used for ATPase assay or calmodulin-binding studies.

Ca^{2+}-ATPase assay. Ca^{2+}-ATPase in red cell membranes was measured as the difference in ATP hydrolysis in the presence and absence of Ca^{2+} (20 μM, established by ion-selective electrode). The details of the assay have been previously published (7). Enzyme activity was expressed as micromoles inorganic phosphate (P_i) per milligram membrane protein per 90 min assay time. Assays were carried out on duplicate samples and results are expressed as the means (±SE) of three or more assays. Intra- and interassay coefficients of variation were ±1% and ±5%, respectively.

Retinoids were dissolved in 95% ethanol and diluted in 10 mM Tris to achieve final concentrations of 10^{-9} to 10^{-6} M in 1% ethanol; con-

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1. *Abbreviations used in this paper:* ANOVA, one-way analysis of variance; ANS, 8-anilino-1-naphthalene sulfonic acid; Ca^{2+}-ATPase, Ca^{2+}-stimulable, Mg^{2+}-dependent adenosine triphosphatase; T_4, L-thyroxine; T_3, 3,5,3'-L-triiodothyronine; W-7, *N*-(6-aminohexyl)-5-chloro-1-naphthalene sulfonamide.

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trol samples received equivalent volumes of diluent. Membranes were exposed to retinoid analogue or diluent, or calmodulin diluted in 10 mM Tris, for 60 min at 37°C before ATPase assay.

Binding of ^{125}I-calmodulin to erythrocyte membranes. Red cell membranes (1.0 mg/ml), retinoic acid or retinol, and tracer ^{125}I-calmodulin, 6×10^{-12} M in 10 mM Tris HCl buffer, were incubated for 60 min at 37°C. The bound/free partition of radiocalmodulin between red cell membranes and buffer was then effected by centrifugation (20,000 $g \times 20$ min), as we have previously described with myocardial membranes (13). In the absence of unlabeled calmodulin or retinoid, membranes bound 9% of tracer (3.3% was specific, and 5.7% nonspecific binding). Unlabeled calmodulin was added in 10 mM Tris in concentrations from 10^{-9} to 10^{-6} M. Retinoic acid and retinol (final concentrations, 10^{-10} to 10^{-6} M) were added to membrane aliquots with a final ethanol concentration of 1%.

Displacement of 8-anilino-1-naphthalene sulfonic acid (ANS) from calmodulin by retinoic acid. The Ca^{2+}-dependent binding of ANS to calmodulin was determined from the increase in fluorescence emission of the dye that accompanies its binding to calmodulin (14, 15). Fluorescence was determined in a spectrofluorometer (SLM 8000; Aminco Corp., Urbana, IL) using an excitation wavelength of 340 nm and scanning fluorescence emission from 400 to 600 nm. Calmodulin (10^{-6} M) was incubated with ANS, 10^{-5} M, in 50 mM Tris HCl, pH 7.4, and EGTA, 1 mM. The Ca^{2+}-dependent binding of ANS to calmodulin was taken as the increase in fluorescence signal associated with addition of $CaCl_2$, 1.5 mM, to the above buffer. ANS displacement was determined in the presence of retinoic acid (concentrations ranging from $1-5 \times 10^{-6}$ M) or diluent (0.1–0.5% ethanol) in control samples.

Assay of calmodulin-dependent cyclic nucleotide phosphodiesterase. Phosphodiesterase activity was determined by the method of Schaeffer et al. (16) with inorganic phosphate determined by the malachite green assay (17). Calmodulin-dependent activity was defined as the difference in enzyme activity in samples lacking and containing calmodulin, 1.2×10^{-8} M, and was expressed as nanomoles P_i/minute. Retinoic acid, or its diluent ethanol in a final concentration of 1%, was added to the assay mixture before initiation of the reaction.

Statistical analysis. Data were analyzed by paired *t* test or one-way analysis of variance (ANOVA).

Results

Red cell Ca^{2+}-ATPase activity; effect of retinoids. Control Ca^{2+}-ATPase activity was 0.387±0.014 μmol P_i per mg protein per 90-min assay period. All *trans*-retinoic acid, 10^{-10} to 10^{-6} M, caused a dose-dependent reduction in enzyme activity (Fig. 1): 26% reduction by 10^{-8} M and 51% by 10^{-6} M ($P < 0.001$, ANOVA). As the figure demonstrates, retinol, retinal, and 13-*cis*-retinoic acid in the same concentrations had no effect on enzyme activity. The benzene ring-substituted analogue of retinoic acid, RO 8-8717, did not inhibit Ca^{2+}-ATPase activity (99% of control enzyme activity in the presence of 10^{-6} M analogue).

Effect of purified calmodulin on membrane Ca^{2+}-ATPase activity in the presence of retinoids. Purified calmodulin, 6×10^{-9} M (100 ng/ml), when added to membranes for 60 min at 37°C, maximally stimulated red cell Ca^{2+}-ATPase activity (Fig. 2). In the presence of 10^{-9} and 10^{-8} M retinoic acid, there was a reduction in maximal enzyme activity achieved with the addition of calmodulin, as well as a shift in the calmodulin dose-response curve to the right; thus, in the presence of 10^{-8} M retinoic acid, maximal stimulation of the enzyme was seen with 1.2×10^{-8} M calmodulin. Retinol had no effect on enzyme stimulation by calmodulin at either concentration.

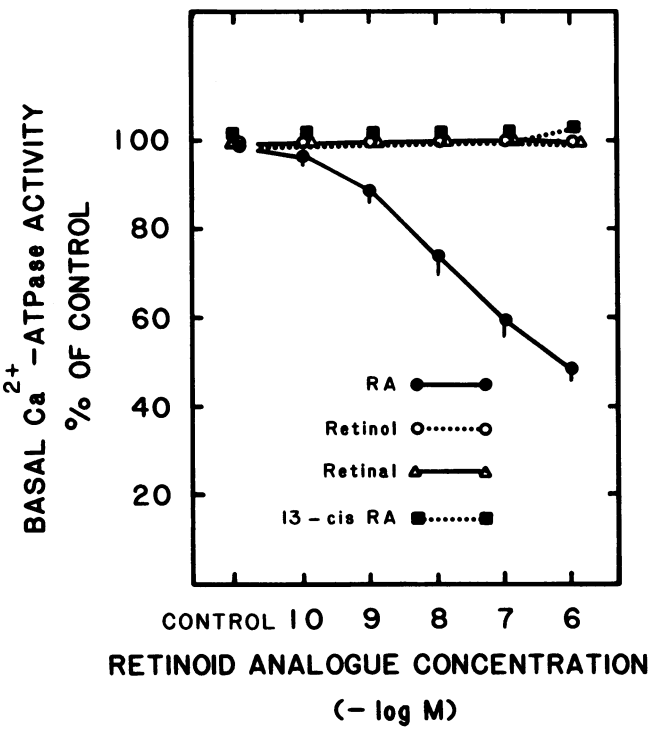

Figure 1. Effect of retinoid analogues on human erythrocyte Ca^{2+}-ATPase activity. Results are expressed as percentage of control enzyme activity without retinoid, and the mean±SE of three experiments performed in duplicate are shown. Retinoic acid (RA) significantly reduced enzyme activity ($P < 0.005$, ANOVA), while retinol, retinal, and 13-*cis* RA were inactive.

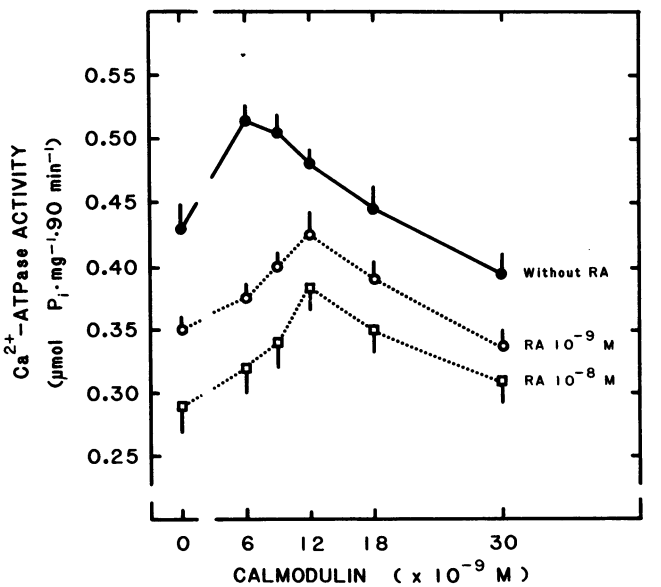

Figure 2. Interaction of retinoic acid and exogenous purified calmodulin on red cell Ca^{2+}-ATPase activity. Results shown are the mean activities from three experiments performed in duplicate. Without RA, calmodulin stimulated enzyme activity, with maximal effect seen at a concentration of 6×10^{-9} M. RA, 10^{-9} and 10^{-8} M, caused dose-related inhibition of enzyme activity and a shift of maximal calmodulin stimulation to the right.

The effect of retinoic acid on binding of calmodulin to red cell membranes. Both retinoic acid and unlabeled calmodulin significantly reduced membrane binding of ^{125}I-calmodulin (6×10^{-12} M, $P < 0.001$, ANOVA), with 50% reduction in binding at concentrations of 1.1 and 2.5×10^{-8} M, respectively (Fig. 3). Nonspecific binding of calmodulin to membranes was 63% of total calmodulin binding, as determined by incubation of ^{125}I-calmodulin in the presence of 10^{-6} M unlabeled calmodulin. Retinol did not displace radiolabeled calmodulin from red cell membranes (data not shown).

ANS-binding to calmodulin in the presence of retinoic acid. The calcium-dependent binding of ANS to calmodulin was accompanied by an increase in fluorescence emission intensity and a shift of the emission maximum from 520 to 500 nm. The fluorescence emission spectrum of calmodulin-bound ANS was virtually unchanged in the presence of 1, 2, and 5 $\times 10^{-6}$ M retinoic acid (Fig. 4, *top*). The slight apparent increase in fluorescence seen at the highest concentration of the retinoid is attributable to light scattering by the sample. In contrast, the addition of identical concentrations of the compound W-7, a well-characterized calmodulin antagonist (18), resulted in a dose-dependent reduction in ANS fluorescence as this agent displaced ANS from calmodulin (30% decrease at 5×10^{-6} M W-7, Fig. 4, *bottom*). Retinoic acid also failed to alter the fluorescence emission spectrum of dansyl-calmodulin (data not shown).

Effect of retinoic acid on phosphodiesterase activity. Phosphodiesterase activity was stimulated threefold by the addition of $1.2 + 10^{-8}$ M calmodulin (7.7 vs. 23.8 nmol P_i/min, control vs. calmodulin). The addition of retinoic acid, 10^{-10} to 10^{-6} M, did not alter the effect of calmodulin (results not shown).

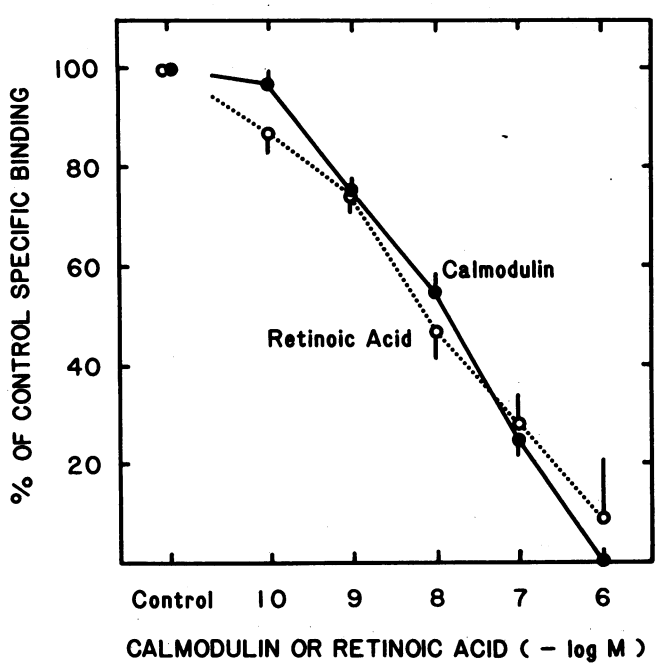

Figure 3. Effect of retinoic acid and unlabeled calmodulin on binding of tracer ^{125}I-calmodulin to red cell membranes. Results are from three experiments performed in duplicate. Displacement of 50% of tracer was obtained with 1.1 and 2.5×10^{-8} M RA and calmodulin, respectively.

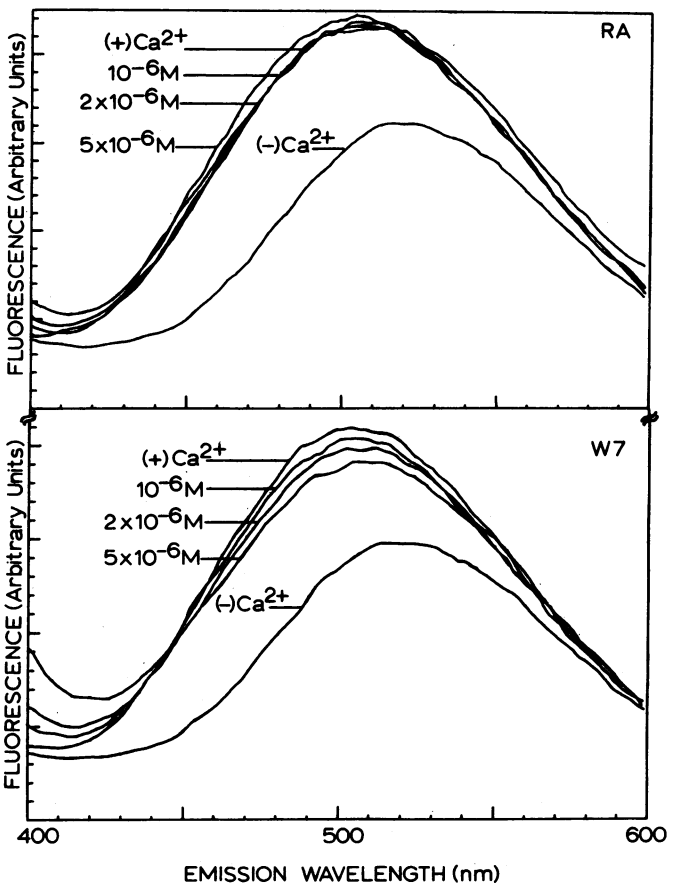

Figure 4. Displacement of calmodulin bound to ANS by retinoic acid, and for comparison, W-7. The addition of $CaCl_2$, 1 mM, caused an increase in fluorescence emission intensity and shift of the curve to the left, compared with the curve in the absence of Ca^{2+}. RA, 1 to 5×10^{-6} M, did not alter the fluorescence emission spectrum of ANS bound to calmodulin (*top*). In contrast, W-7, a known calmodulin antagonist, caused a dose-dependent reduction in ANS fluorescence (bottom).

Discussion

Retinoids have important roles in the regulation of growth and differentiation in normal and transformed epithelial and nonepithelial cells (19, 20). The mechanisms of the actions of retinoic acid and its parent, retinol, appear to be nuclear (21, 22) as well as extranuclear (23). In the present studies we have shown that retinoic acid, but not retinol, is a "calmodulin antagonist" in the context of the activation of human red cell Ca^{2+}-ATPase by calmodulin. The evidence for the anti-calmodulin action of this retinoid includes (*a*) reduction of basal activity of Ca^{2+}-ATPase, presumably reflecting antagonism of residual endogenous calmodulin bound to red cell ghosts (9), (*b*) an alteration in the V_{max} for calmodulin stimulation in the presence of inhibitory concentrations of retinoic acid and (*c*) competition between retinoic acid and calmodulin for human red cell membrane binding sites.

Studies of the mechanism by which retinoic acid inhibits calmodulin's stimulation of Ca^{2+}-ATPase, however, revealed that the retinoid was not a conventional calmodulin antagonist in that it did not directly interact with this Ca^{2+}-binding

protein. Spectrofluorometric assessment of ANS-binding to purified calmodulin indicated that retinoic acid and ANS did not compete for sites on that protein and stimulation by calmodulin of purified cyclic nucleotide phosphodiesterase activity was not inhibited by retinoic acid. Several laboratories, including our own, have shown that ANS interacts with calmodulin at more than one site (15, 24, 25), including the hydrophobic region on the Ca^{2+}-binding protein that is critical to phosphodiesterase and plasma membrane Ca^{2+}-ATPase activity. Thus, the ANS-calmodulin model was an appropriate one with which to determine whether the calmodulin antagonism of retinoic acid reflected a direct interaction of the retinoid with calmodulin. Since retinoic acid did not affect the ANS-calmodulin complex, but inhibited calmodulin's binding to red cell membranes, we concluded that the inhibition by retinoic acid of calmodulin's stimulation of Ca^{2+}-ATPase is membrane-mediated, at the membrane calmodulin-binding site involved in Ca^{2+}-ATPase stimulation. The fact that retinol, retinal, and 13-*cis* retinoic acid had no anticalmodulin action supports the biologic specificity of action of retinoic acid at the plasma membrane, and furthermore attests to the divergence of actions of retinol and retinoic acid recognized previously, related to spermatogenesis and vision (26, 27).

Evidence for the existence of a membrane calmodulin binding site has been developed in our laboratory (13) and by others (2, 28). The possibility has not been excluded, however, that retinoic acid can also affect Ca^{2+}-ATPase by an interaction with the plasma membrane separate from the calmodulin-binding site since other studies from our laboratory have shown that retinoic acid (but not retinol), competes for plasma membrane binding sites with thyroid hormone (12).

The significance of the action of retinoic acid on calmodulin's stimulation of red cell membrane Ca^{2+}-ATPase activity is twofold. First, these observations support a novel model of calmodulin antagonism that involves a membrane calmodulin-binding site, rather than interaction of the antagonist directly with calmodulin itself. This mechanism also appears to be involved in the inhibition by specific long chain fatty acids of the stimulation of Ca^{2+}-ATPase by calmodulin (29). The benzene ring-substituted analogue of all-*trans* retinoic acid does not inhibit Ca^{2+}-ATPase activity, however, indicating a specific inhibitory effect of the retinoid structure; the effect is not simply a reflection of the fatty acid side-chain of retinoic acid. Second, retinoic acid has discrete actions at the plasma membrane, in addition to those that are genomic and involve the superfamily of nuclear receptors for corticosteroids, thyroid hormone, and the retinoid (30).

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