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In Vitro Effect of Retinoids on Calcineurin Activity

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ABSTRACT. Calcineurin was shown previously to be inhibited by members of the tyrphostin family of tyrosine kinase inhibitors, with the most effective inhibition suggested to be caused by the presence of a conjugated side chain (Martin BL, *Biochem Pharmacol* 56: 483–488, 1998). Retinoids are a family of naturally occurring biomolecules having non-aromatic ring structures and conjugated side chains as substituents on the ring. Three oxidation states of the all-*trans* configuration of retinoids (retinol, retinal, and retinoic acid) were tested as effectors of calcineurin. Only retinoic acid was found to inhibit calcineurin effectively, with an IC_{50} value of approximately 50 μM. Retinol and retinal caused less than 30% inhibition at concentrations up to 100 μM. All three retinoids caused some precipitation of reaction components: retinoic acid and retinal above 50 μM, and retinol above 250 μM. Bacterial alkaline phosphatase was not inhibited by the retinoids, indicating that metal centers alone are insufficient for significant inhibition by retinoic acid. An aromatic ring was not required for inhibition and may not provide additional inhibition, inasmuch as an aromatic analog of retinoic acid (acitretin) showed less effective inhibition. These data are consistent with the presence of conjugated, unsaturated groups enhancing the inhibition of calcineurin. BIOCHEM PHARMACOL **60**;6:803–808, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. calcineurin; calmodulin-activated phosphatase; retinoids

The calmodulin-activated phosphatase calcineurin was identified as the cellular target for immunosuppressants [1, 2] typified by cyclosporin A complexed with its receptor cyclophilin. Subsequently, cyclosporin A (or FK506) was used as a reagent for further definition of calcineurin substrates and biological functions [3–8]. There are difficulties, however, when assigning physiological functions of calcineurin using cyclosporin A as a cellular probe. For example, calcineurin is inhibited only partially despite levels of cyclosporin expected to inhibit the enzyme completely [9], but the inhibition of calcineurin does seem to be critical for the biological effects of cyclosporin. Such difficulties may possibly be overcome by using a mechanism-based inhibitor of the enzyme, highlighting the need for the characterization of calcineurin.

Initial studies focused on compounds resembling tyrosine as an analog for the small substrate tyrosine phosphate. Some members of the tyrphostin family of tyrosine kinase inhibitors were found to be inhibitors of calcineurin [10] with inhibition occurring at concentrations similar to concentrations needed for the inhibition of tyrosine kinases. Inhibition seemingly was enhanced by the presence of unsaturated and conjugated side chains as a substituent on the aromatic ring of the tyrphostin. Tyrphostins having a saturated side chain or less conjugation were not effective inhibitors. The aromatic ring portions of the molecules were similar, suggesting a less important role for this part of the molecule. It was concluded that the conjugated side chain was important for inhibition of calcineurin, possibly through interactions with some electron-deficient area on the enzyme. The relative contributions of the aromatic ring and the unsaturated side chain could not be distinguished.

One group of biologically active molecules containing conjugated side chains are the retinoids. These vitamin A metabolites have a non-aromatic ring with a terpene side chain with 4 carbon—carbon double bonds. Members of this class of biomolecules were tested as effectors of calcineurin. The variants tested were all-*trans* retinol, all-*trans* retinal, and all-*trans* retinoic acid, comprising all three oxidation states. Only retinoic acid, however, can fully ionize and optimize conjugation within the side chain.

MATERIALS AND METHODS

Materials

The substrate pNPP§ (Sigma 104 substrate) was purchased from the Sigma Chemical Co. as were EGTA, MOPS, and phenyl-Sepharose. The retinoids tested as effectors were also purchased as the all-*trans* configurations from the Sigma Chemical Co. The aromatic retinoid acitretin (Soriatane or all-*trans*-9-(4-methoxy-2,3,6-trimethylphenyl)-

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§ *Abbreviations*: IC_{50}, concentration resulting in inhibition of 50% of phosphatase activity; MOPS, 3-(*N*-morpholino)propanesulfonic acid; pNPP, *para*-nitrophenyl phosphate; and pNP, *para*-nitrophenol.

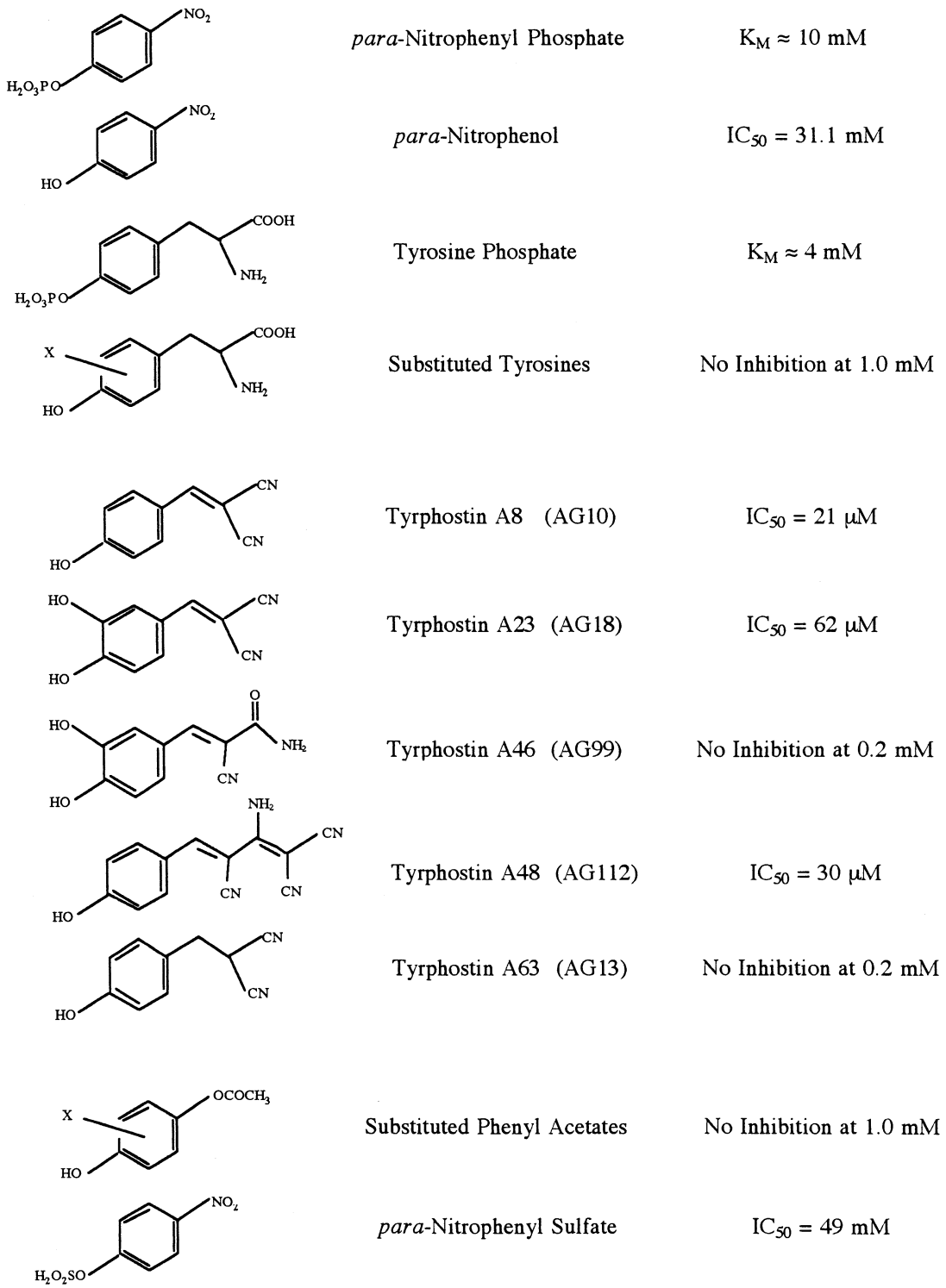

FIG. 1. Structures of the aromatic substrates and inhibitors of calcineurin. The structures of the different compounds tested as substrates and inhibitors of calcineurin are shown. Also provided are estimates for kinetic constants for these compounds.

3,7-dimethyl-2,4,6,8-nonatetraenoic acid) was a gift from Roche Pharmaceuticals. Other chemicals (e.g. metal salts) were obtained from Aldrich Chemical or Fisher Scientific. Chelex-100 was purchased from Bio-Rad Laboratories and was used to remove metal ions from water and buffers used in these experiments.

Proteins

Calcineurin was isolated from bovine brain by the method of Sharma *et al.* [11] except that MOPS buffers were used in place of Tris buffers. Calcineurin purified with this protocol requires Mn^{2+} or Ni^{2+} for full activity [12–14] and is not

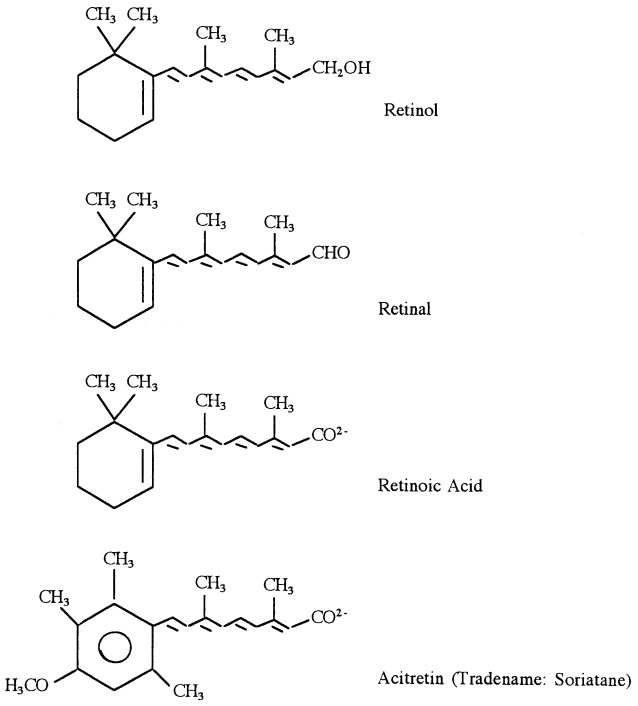

FIG. 2. Structures of retinoids. The structures of the different retinoids used are shown. The base family of retinoids are non-aromatic ring structures, whereas acitretin is an aromatic retinoid.

activated further by the addition of $CaCl_2$. Calmodulin was purified by the procedure of Sharma and Wang [15] with a slight modification. Following DE-52 chromatography, the calmodulin was loaded onto phenyl-Sepharose and eluted with EGTA [16]. Protein concentrations were determined by the method of Bradford [17].

Phosphatase Assays

Calcineurin was assayed by measuring the release of pNP spectrophotometrically at 410 nm in a Spectramax 250 microtiter plate reader (Molecular Devices) with a total volume of 200 mL. The standard assay for characterization of activity was done at 30° in 25 mM MOPS, pH 7.0; 1.0 mM $MnCl_2$; 2–5 μg/mL of calmodulin; and 2–5 μg/mL of calcineurin with 10 mM pNPP as the substrate. Retinoids were prepared as 10 mM stocks in 80% DMSO with final reaction solutions containing 2% DMSO. Alkaline phosphatase (6 μg/mL) was assayed using similar conditions, except that 25 mM Bicine, pH 9.0, was used in place of the MOPS buffer, and $MgCl_2$ was included at 0.1 mM instead of $MnCl_2$. Stock solutions of the retinoids were prepared in DMSO. DMSO was added to reaction mixtures to maintain a constant concentration at 2% (v/v).

RESULTS AND DISCUSSION

Various compounds have been used as inhibitors of calcineurin. In all cases, the putative inhibitors contain an aromatic ring owing to the structural similarity to pNPP and tyrosine phosphate, artificial substrates of calcineurin. Shown in Fig. 1 are the structures and inhibitory potency for a number of these compounds. Only simple inhibitors are included. Other more complex inhibitors have been identified. Of course, cyclosporin A [2, 18] is a more potent inhibitor, but this inhibitor also requires a specific binding protein [18]. The multi-ring pyrethroids have also been identified as more potent inhibitors [19], although the inhibition has been questioned [20]. For simple structures, however, the tyrphostins seem to be among the best inhibitors reported.

Inhibition by Retinoids

Retinoids are a series of vitamin A derivatives implicated as effectors in various biological signaling events and are often used in the treatment of dermatological conditions, including acne and psoriasis. Retinoids (Fig. 2) contain conjugated side chains as substituents of a non-aromatic ring. Retinoids may be suitable candidates for testing as calcineurin inhibitors. First, conjugated side chains have been suggested to favor enhanced inhibition of calcineurin by tyrphostins. Second, the possible functional overlap between retinoids and cyclosporin A raises the possibility that the biological effects of retinoids may include actions on calcineurin. Experiments were done to evaluate whether retinoids could affect the activity of calcineurin.

Figure 3 shows the inhibition observed with all-*trans*

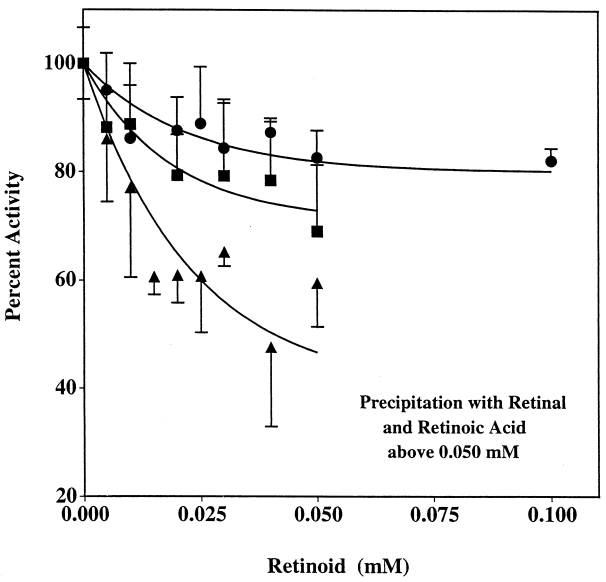

FIG. 3. Inhibition of calcineurin by non-aromatic retinoids. As described in Materials and Methods, calcineurin was assayed using 10.0 mM pNPP as a substrate. Retinoids were added at the indicated concentrations, and calcineurin activity was measured by monitoring the increase in the product, pNP. The retinoids evaluated as inhibitors were retinol (circles), retinal (squares), and retinoic acid (triangles). The control activity (100%) was 0.46 μmol/min/mg of calcineurin under the conditions of the experiment. Each point represents the average of at least three experiments with each experimental measurement done in duplicate. Error bars represent standard deviations.

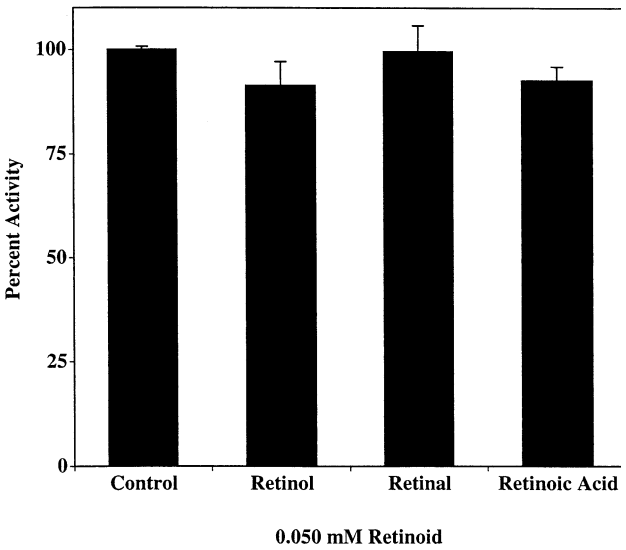

FIG. 4. Inhibition of alkaline phosphatase by non-aromatic retinoids. The effects of the three retinoids on the hydrolysis of 10.0 mM pNPP by *E. coli* alkaline phosphatase were measured. Each retinoid was present at 0.050 mM, approximately the IC_{50} for retinoic acid for inhibition of calcineurin. The control activity (100%) for alkaline phosphatase was 1.86 μmol/min/mg of enzyme. Other conditions are provided in Materials and Methods. Each activity was measured in duplicate, with the error bars representative of the range of the measurements.

forms of retinol, retinal, and retinoic acid. Clearly, retinoic acid was the most effective, with virtually no difference observed between retinol and retinal. The IC_{50} value was estimated as approximately 0.041 mM for retinoic acid, but this value is uncertain because of precipitation in the reaction mixtures when the concentration of retinoic acid was raised above 0.050 mM. The precipitation was observed in the absence of substrate (not shown) so switching substrates would not likely preclude this problem. No IC_{50} could be estimated for retinol and retinal because the observed inhibition was insufficient. Precipitation was also observed with these retinoids, above 0.050 mM for retinal and 0.10 mM for retinol. It seems likely that the more effective inhibition by retinoic acid was because of the presence of the ionizable carboxylic acid group present in retinoic acid. For comparison, these retinoids were tested as inhibitors of alkaline phosphatase from *Escherichia coli*, using a similar assay. No significant inhibition (Fig. 4) of alkaline phosphatase was observed with a 50 μM concentration of each retinoid. There was approximately 8% inhibition caused by retinol, <1% by retinal, and approximately 7% by retinoic acid compared with 17, 31, and 40% inhibition of calcineurin by 50 μM retinol, retinal, or retinoic acid, respectively. These data suggest that there is selectivity in recognition of these inhibitors, although complete understanding of phosphatase specificity will require additional study.

Inhibition by an Aromatic Retinoid

One concern was the absence of the aromatic group in retinoids, but its presence in the tyrphostins (compare Figs. 1 and 2), which may enhance recognition of small molecules by calcineurin. A sample of an aromatic retinoid, acitretin, was obtained and tested as an inhibitor. As shown in Fig. 5, acitretin was a weak inhibitor of calcineurin with an IC_{50} value estimated as 0.35 mM. Unlike the other retinoids, no precipitation was observed with acitretin. The presence of the aromatic group did not provide any additional potency as an inhibitor. The weaker inhibition of acitretin was attributed to the ring substituents, notably the methoxy and methyl groups, which likely contributed steric interference to effective binding. As with the data using the tyrphostin family of inhibitors, the conjugated side chain was concluded to be the predominant feature causing inhibition. This is consistent with electron delocalization within the side chain being critical for interaction and inhibition, although the nature of the interaction remains unknown. Interactions with electron-rich protein residues, or with the bound metal ions, remain likely possibilities.

Ring Compared with Unsaturated and Conjugated Side Chain

The inhibition observed with retinoic acid was comparable to inhibition observed with tyrphostins A8 and A23. As with tyrphostins A46, A48, and A63 [10], the weaker inhibition by the aromatic retinoid showed that the presence of an aromatic ring was not sufficient to achieve effective binding and inhibition. These data collectively

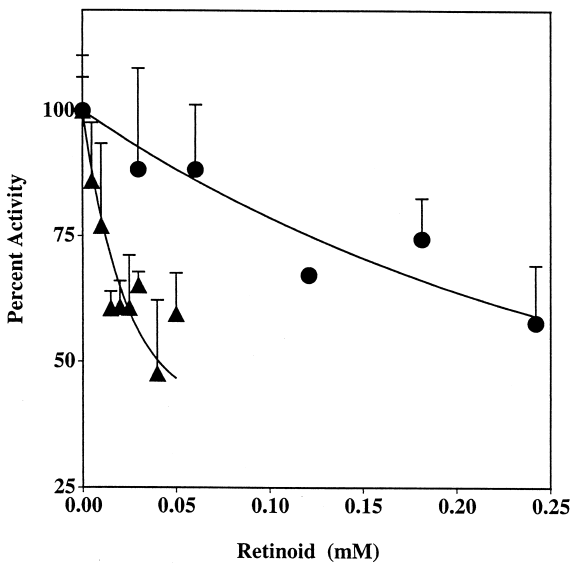

FIG. 5. Inhibition of calcineurin by the aromatic retinoid acitretin. The effect of acitretin on calcineurin was determined as for the other retinoids (Fig. 3 and Materials and Methods). Acitretin (circles) was added at the indicated concentrations, and calcineurin activity was measured. The control activity (100%) was 0.44 μmol/min/mg of calcineurin for the experiments with Soriatane. Each point for acitretin inhibition represents the average of three determinations. Error bars represent the standard deviations for the measurements. The data for inhibition by retinoic acid (triangles; from Fig. 3) are shown for comparison.

are consistent with an important, even predominant, role for the conjugated side chain of the tested inhibitors. The majority of small molecules shown in Fig. 2 all were found to bind weakly and do not have unsaturated side chains. These data are consistent with binding to calcineurin being most effective by small compounds having conjugated side chains. Such a binding motif also likely explains the ability of calcineurin to bind and hydrolyze phospho*enol*pyruvate [13], the only non-aromatic, simple phosphate ester reported to be a substrate for calcineurin.

Potential Significance of Retinoid Inhibition

The inhibition observed with retinoids and tyrphostins may be noteworthy inasmuch as both types of compounds and cyclosporin A, a commonly used calcineurin inhibitor, have been used in similar biological situations, notably in the treatment of psoriasis and psoriatic arthritis [21–23]. In each of these situations, compounds that inhibit calcineurin were found to reduce or block cellular effects associated with psoriasis or psoriatic arthritis. Retinoids have been reported to inhibit other arthritis models such as collagen-induced arthritis induced in rats [24]. Cyclosporin [25] and retinoids [26] have been used in rheumatoid arthritis with similar results. Protein phosphorylation has been implicated as one possible mechanism of signaling in each of these events (psoriasis [27–29], rheumatoid arthritis [30], and a model for collagen-induced arthritis [31]), although the relationship between these inhibitors and protein phosphorylation is not clear. Also calcium-dependent, the activity of phosphorylase kinase has been found to be increased in psoriatic epidermal cells [32, 33]. In HL-60 cells, however, retinoic acid has been reported to cause increased levels of calcineurin activity, although the increase was attributed to increased expression of the protein subunits [34]. No direct effect on the calcineurin molecule was assigned to retinoic acid.

Extrapolation of the significance of these data to cellular events is also difficult because of the role of retinoid binding proteins, which were not included in any reaction mixture. Retinoid binding proteins function to carry different forms of retinoids in the circulatory system and to target functions of the retinoid within the cell. As cyclophilins act as binding proteins for cyclosporin A and are required for inhibition of calcineurin, retinoid binding protein may be needed to achieve full effects. The relative failure of retinol and retinal may be because these forms require the binding protein. There are differences inasmuch as cyclosporin A shows an all-or-nothing requirement for a binding protein to achieve inhibition. Clearly, retinoic acid did not show a similar dependency.

In this investigation, the retinoids were studied primarily as model compounds having a conjugated side chain. These data provide only a starting point for the future development of calcineurin inhibitors. The presence of an unsaturated and conjugated chain (side chain) would provide an initial pharmacophore to develop calcineurin inhibitors.

Special acknowledgment is given to Roche Pharmaceuticals for providing a sample of acitretin for these studies.

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