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Inhibition of calcineurin by polyunsaturated lipids

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Abstract

From earlier studies on calcineurin, the presence of multiple double bonds in putative inhibitors was hypothesized as critical features for effective inhibition. Polyunsaturated fatty acids were tested as inhibitors of calcineurin and found to inhibit the phosphatase activity of calcineurin although effective inhibition was observed only in the absence of calmodulin. Calmodulin and fatty acids seemed to compete for the enzyme with the activation curve of calmodulin shifted approximately 100-fold in the presence of 50 μM eicosa-11*Z*,14*Z*-dienoic acid (20:2, *n*-6) or 50 μM eicosa-8*Z*,11*Z*, 14*Z*-trienoic acid (20:3, *n*-6). Leukotriene B4 and derivatives also were screened as inhibitors. The most effective inhibition was caused by the 6-*trans*,12-epi-leukotriene B4 with an IC_{50} of 16.4 μM for the inhibition of calcineurin with pNPP as the substrate. Lipoxins A4 and B4 likewise caused inhibition in the presence of calmodulin with an IC_{50} of 42.7 μM for lipoxin B4. There was no protection by calmodulin, as found with the inhibition by the fatty acids. These data support the hypothesis that effective inhibition is bolstered by the presence of conjugated double bonds in the inhibitor. Consideration of *cis*- and *trans*-orientation of the double bonds suggests that presentation of the delocalized electron density is also a factor in effective inhibition of calcineurin.

Keywords: Calcineurin; Calmodulin activation; Fatty acids; Polyunsaturated; Inhibitor specificity

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1. Introduction

With the demonstration that the calcineurin was the target of immunosuppressants used in transplantation [1,2], these drugs have been used to establish that numerous physiological processes involve calcineurin. Using the cyclosporin-A as an inhibitor, calcineurin was found to regulate gene expression in the development of cardiac hypertrophy [3–5]. Unfortunately, immunosuppressant drugs also have deleterious side effects in a significant pool of patients, notably nephrotoxicity. Examination of the characteristics of calcineurin inhibitors was begun with the goal of developing selective calcineurin inhibitors that lack the toxic side effects of cyclosporin-A. Based on early studies with small substrates, tyrosine derivatives were first tested as possible inhibitors of calcineurin [6] and found to be weak inhibitors. Despite this, the inability of calcineurin to hydrolyze free phosphoserine or free phosphothreonine indicated that the aromatic structure may be critical for recognition. One set of aromatic candidates tested as possible calcineurin inhibitors was the tyrphostin family of tyrosine kinase inhibitors [7]. Differences in inhibition were found with select tyrphostins and seemed related to the structures of the inhibitors, particularly regarding the conjugation of unsaturated bonds in the side chains of the inhibitors. The effect of bond conjugation was most apparent in the difference between two tyrphostins, A8 and A63, that are identical except for the double bond between C_α and C_β of the aliphatic chain. Tyrphostin A8, containing the double bond, inhibited calcineurin whereas A63 did not. The unsaturated aliphatic group enabled conjugation along the entire backbone with electron density delocalized throughout the structure.

From these data, it was not apparent whether the aromatic ring or the conjugated side chain was the predominant structural feature responsible for the inhibition. To begin to distinguish between these features, retinoids were evaluated as inhibitors [8]. Retinoids are naturally occurring biomolecules having conjugated side chains as substituents on a non-aromatic ring. Three oxidation states of the *all-trans* configuration of retinoids (retinol, retinal, and retinoic acid) were tested. Only retinoic acid was found to effectively inhibit calcineurin with an IC_{50} value of approximately 50 μM with retinol and retinal causing less than 30% inhibition at equivalent concentrations. Bacterial alkaline phosphatase was not inhibited by the retinoids indicating that a metal ion center alone was insufficient for significant inhibition by retinoic acid. These data did suggest that an aromatic ring was not an absolute requirement for inhibition. An aromatic analog of retinoic acid (acitretin) was tested and caused less inhibition than retinoic acid; the IC_{50} for acitretin was 350 μM. The aromatic ring did not account for additional functional inhibition. These data were consistent with enhanced inhibition being supported by the presence of regions of electron delocalization. Structures and inhibition data for the described compounds are shown in Fig. 1.

Because there was reason to conclude that the aromatic ring was less critical for effective inhibition, we aimed to characterize the effect of bioactive polyunsaturated aliphatic molecules. In this report, polyunsaturated lipid molecules were tested as inhibitors. Based on the hypothesis that unsaturated bonds enhance inhibition, it was expected that all compounds would cause inhibition.

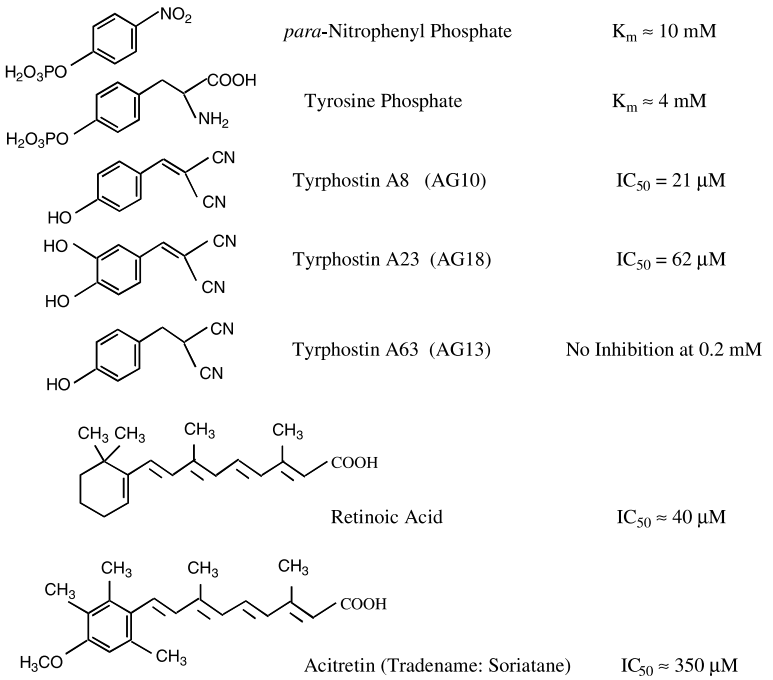

Fig. 1. Inhibitors of calcineurin. Shown are the structures and IC_{50} values for compounds previously characterized.

2. Experimental procedures

2.1. Materials

Buffers, EGTA,[1] DMSO, and phenyl-sepharose were purchased from Sigma–Aldrich (St. Louis, MO). The substrate *p*NPP (Sigma 104 substrate) and saturated fatty acids were also purchased from Sigma–Aldrich. DE-52 cellulose was obtained from Whatman (Florham Park, New Jersey). All polyunsaturated fatty acids were obtained from Biomol Research Laboratories (Plymouth Meeting, PA) as were lipoxin A4 and lipoxin B4. Leukotriene B4 and isomers were purchased from Cayman Chemical (Ann Arbor, MI). Except for the saturated fatty acids, all lipids were obtained as ethanol solutions. Other chemicals (metal salts, etc.) were obtained from Fisher (Pittsburgh, PA). All solutions were prepared from water that had been treated with Chelex-100.

2.2. Proteins

Bovine brain calcineurin was isolated from bovine brain to apparent homogeneity using a modification of the method of Sharma et al. [9] using MOPS buffer instead of Tris for the

[1] *Abbreviations used:* CaM, calmodulin; EGTA, ethylene glycol bis(β-amino ethyl ether)*N*,*N*′-tetraacetate; LTB4, leukotriene B4; LXA4, lipoxin A4; LXB4, lipoxin B4; MOPS, 3-(*N*-morpholino)propanesulfonic acid; *p*NP, *para*-nitrophenol; *p*NPP, *para*-nitrophenyl phosphate.

preparation of all solutions. Calmodulin was purified by the procedure of Sharma and Wang [10] but including chromatography on phenyl-sepharose with elution by EGTA [11]. Protein concentrations were determined by the method of Bradford [12]. Purified bovine brain calcineurin also was obtained from Upstate Biotechnology (Charlottesville, VA).

2.3. Calcineurin assay and analysis of kinetic data

Activity measurements were done at 30 °C with standard assay conditions of 10 mM *p*NPP in 25 mM MOPS, pH 7.0; 5 μg/ml calmodulin; 2.5 μg/ml calcineurin with 1.0 mM Mn^{2+}. In some experiments, calmodulin was varied from 2 to 20 μg/ml. Fatty acid solutions were prepared in ethanol at 0.1 M concentrations. For use, the fatty acids were diluted to 10 mM in DMSO. For the leukotrienes and lipoxins, the ethanol was evaporated under a stream of filtered nitrogen, and the lipids dissolved in dimethyl sulfoxide as stock solutions. For all assays, dimethyl sulfoxide was included to a final concentration of 10% (v/v). The reactions with *p*NPP as the substrate were monitored continuously at 410 nm on a Beckman DU7400 spectrophotometer or a Spectromax model 384 microtiter plate reader. The microtiter plate reader has a monochromator for selecting wavelengths in 1 nm increments. Data fitting and numerical estimates were done using the programs Deltagraph (SPSS; Chicago, IL) and Prism (GraphPad; San Diego, CA).

3. Results and discussion

3.1. Inhibition by polyunsaturated fatty acids

Inhibition of calcineurin by polyunsaturated fatty acids was compared at a concentration of 50 μM and all fatty acids were found to inhibit to some extent (Table 1). Greater inhibition was observed in the absence of calmodulin consistent with an earlier report [13]. With calmodulin included, the maximal inhibition observed was 43% with eicosa-11*Z*,14*Z*,17*Z*-trienoic (20:3, *n*-3), but the inhibition by this fatty acid was 87% without calmodulin included. These data showed that the fatty acids could directly inhibit calcineurin and did not require the presence of calmodulin. This observation provides a critical difference from the inhibition observed with tyrphostins and retinoids [7,8]. Tyrphostins and retinoic acid caused significant inhibition at 50 μM when calmodulin was included in the assay mixture and there was no evidence for protection by calmodulin.

There was little difference between the fatty acids as inhibitors in the presence of calmodulin. Three of the polyunsaturated fatty acids, eicosa-11*Z*,14*Z*-dienoic acid (20:2, *n*-6), eicosa-8*Z*,11*Z*,14*Z*-trienoic acid (20:3, *n*-6), and eicosa-5*Z*,8*Z*,11*Z*,14*Z*-tetraenoic acid (arachidonic acid, 20:4, *n*-6), also were evaluated at a concentration 100 μM. For these three fatty acids, increasing the concentration of the 100 μM did not cause a significant increase in inhibition (Fig. 2). These data suggested that increasing the number of double bonds did not cause elevated inhibition with calmodulin included.

Inhibition was observed with both omega-3 and omega-6 families of fatty acids. With calmodulin included, there was little difference between the omega-3 and omega-6 families. Without calmodulin, the omega-3 fatty acids (averaging 80% inhibition) were slightly more effective as a group than the omega-6 fatty acids (averaging 71% inhibition), suggesting a hint of the influence of the positions of the double bonds. There was also some indication that the number of double bonds did have a limited influence on inhibition. For example, with the eicosa-

Table 1
Inhibition of calcineurin by fatty acids

Fatty acid	Designation	Percent remaining activity	
		+CaM	−CaM
None		100 ± 6	100 ± 7
Octadecanoic	18:0	112 ± 10	203 ± 16
Eicosanoic	20:0	113 ± 8	211 ± 21
Docosanoic	22:0	110 ± 5	161 ± 6
Octadeca-9*Z*,12*Z*-dienoic	18:2, *n*-6	90 ± 9	25 ± 4
Octadeca-9*Z*,12*Z*,15*Z*-trienoic	18:3, *n*-3	81 ± 12	18 ± 1
Octadeca-6*Z*,9*Z*,12*Z*-trienoic	18:3, *n*-6	104 ± 3	47 ± 2
Eicosa-11*Z*,14*Z*-dienoic	20:2, *n*-6	86 ± 4	11 ± 1
Eicosa-11*Z*,14*Z*,17*Z*-trienoic	20:3, *n*-3	57 ± 8	13 ± 2
Eicosa-8*Z*,11*Z*,14*Z*-trienoic	20:3, *n*-6	78 ± 7	36 ± 4
Eicosa-5*Z*,8*Z*,11*Z*-trienoic	20:3, *n*-9	63 ± 12	9 ± 2
Eicosa-5*Z*,8*Z*,11*Z*,14*Z*-tetraenoic	20:4, *n*-6	90 ± 4	37 ± 12
Eicosa-5*Z*,8*Z*,11*Z*,14*Z*,17*Z*-pentaenoic	20:5, *n*-3	90 ± 4	41 ± 3
Docosa-13*Z*,16*Z*,19*Z*-trienoic	22:3, *n*-3	66 ± 19	20 ± 2
Docosa-7*Z*,10*Z*,13*Z*,16*Z*-tetraenoic	22:4, *n*-6	67 ± 17	18 ± 7
Docosa-7*Z*,10*Z*,13*Z*,16*Z*,19*Z*-pentaenoic	22:5, *n*-3	80 ± 6	15 ± 3
Docosa-4*Z*,7*Z*,10*Z*,13*Z*,16*Z*,19*Z*-hexaenoic	22:6, *n*-3	100 ± 17	15 ± 1

Each fatty acid was present at 50 μM. When included, calmodulin was present at 5 μg/ml. Each value represents the average of 3–6 determinations with the standard deviation shown for the measurement.

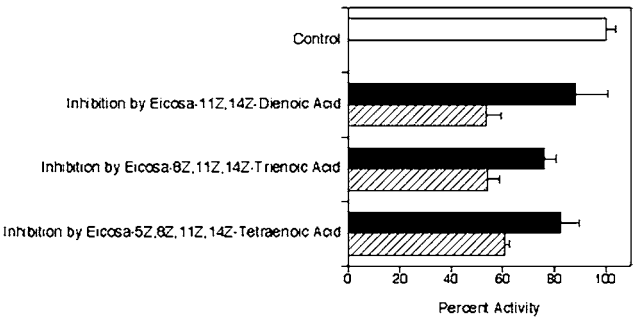

Fig. 2. Calmodulin protection of fatty acid inhibition of calcineurin. Shown is a comparison for the inhibition of calcineurin by 3 omega-6 polyunsaturated fatty acids at 50 μM (filled bars) and 100 μM (hatched bars) in the presence of 5 μg/ml calmodulin. Activity was measured using *p*NPP as the substrate and was compared to activity without added fatty acid (open bar). Each bar represents the average of three measurements with the error bar showing the standard deviation of the measurements.

omega-6 fatty acids, inhibition was greater with only 2 double bonds (89% inhibition) compared to 3 (64%) or 4 (63%) double bonds. For the eicosa-omega-3 fatty acids, a similar effect was found with 3 double bonds (87% inhibition) compared to 5 double bonds (59% inhibition). A similar effect was not seen with the docosa-fatty acids; all caused similar inhibition regardless of the number of double bonds. The effects of increasing the number of double bonds may be related to the flexibility of the molecule. Increasing the number of double bonds will increase the rigidity of the structure and may make it less able to interact with the protein.

Saturated fatty acids of comparable length (18:0, 20:0, and 22:0) were also tested as inhibitors. No inhibition was observed; in fact, calcineurin activity was increased with 50 μM saturated fatty acid added to the assay mix (Table 1). The inclusion of calmodulin

prevented the significant activation by the saturated fatty acids, although there was evidence for slight activation even in the presence of calmodulin. These data indicate differences between polyunsaturated and saturated fatty acids on the activity level of calcineurin. A common feature, however, was the protective effect of calmodulin modulating both the inhibition and activation by the fatty acids.

3.2. Fatty acids and calmodulin activation

Because of the more effective inhibition in the absence of calmodulin, it was concluded that the polyunsaturated fatty acids caused of inhibition of calcineurin by binding directly to calcineurin and not to calmodulin. The apparent ability of calmodulin to block inhibition suggested that calmodulin and the fatty acids may compete for active calcineurin. Experiments showed that the calmodulin activation curve was shifted in the presence of fatty acid inhibitors (Fig. 3). The estimated value of K_{act} for calmodulin was shifted approximately 50- to 100-fold in the presence of an inhibitor. In the absence of fatty acid, K_{act} was approximately 0.090 ± 0.003 μg/ml. This value was shifted to 4.5 ± 2.5 and 8.8 ± 3.9 μg/ml in the presence of eicosa-11*Z*,14*Z*-dienoic acid (20:2, *n*-6) and eicosa-8*Z*,11*Z*,14*Z*-trienoic acid (20:3, *n*-6), respectively. These data indicate that fatty acid did weaken the calcineurin–calmodulin interaction. It was postulated that the calmodulin binding sequence of calcineurin may share sequence relationship with fatty acid binding proteins. A BLAST search failed to identify any sequence relationship between calcineurin and fatty acid binding proteins.

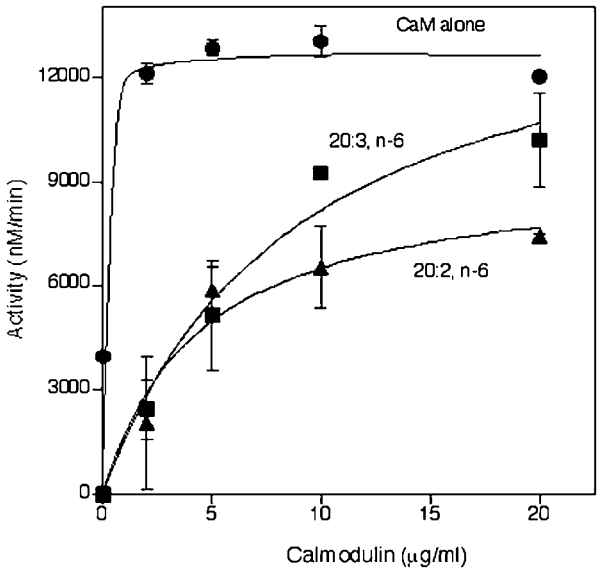

Fig. 3. Competition between fatty acids and calmodulin. Shown is the response curve of calcineurin to increasing concentrations of calmodulin alone (circles), or increasing concentrations of calmodulin in the presence of 50 μM eicosa-11*Z*,14*Z*-dienoic acid (20:2, *n*-6; triangles) or increasing calmodulin in the presence of 50 μM eicosa-8*Z*,11*Z*,14*Z*-trienoic acid (20:3, *n*-6; squares). Activity was measured using *p*NPP as the substrate. Each data point is the average of three determinations with the error bars showing the standard deviation of the measurement. For some data points, the error bar is smaller than the size of the symbol.

3.3. Comparison to reported effects of fatty acids on calcineurin

Our observations showed differences from those reported by Kessen et al. [13], who found activation of calcineurin by unsaturated fatty acids independent of the presence of calmodulin. There was no apparent activation by saturated fatty acids or by the methyl ester of arachidonic acid. These authors showed that arachidonic acid caused the dissociation of calcineurin from immobilized calmodulin. Similar experiments were not done in this work because the focus was the development of inhibitors. Instead, we characterized the kinetic effect of the effect of fatty acids and demonstrated that the activation curve for calmodulin was shifted. The activation constant for calmodulin was increased 50- to 100-fold.

Kessen et al. [13] concluded that the fatty acids mimicked the action of calmodulin by binding at the calmodulin site, although no inhibition of calmodulin activation was reported. Likewise, the apparent inhibition reported here could be attributed to binding at the calmodulin site. It is uncertain why the observations differ, but the study of Kessen et al. [13] was done using recombinant catalytic subunit (subunit A, CaN-A) from *Dictyostelium discoideum* contrasted with the present work done with the intact bovine brain enzyme. For the *Dictyostelium* enzyme, there was no apparent need for the regulatory subunit (subunit B, CaN-B) when *p*NPP was used as the substrate. With a phosphopeptide substrate, there was no additional activation by fatty acids in the presence of CaN-B. CaN-A from *Dictyostelium* does differ from the bovine brain enzyme in having extensions at the termini of the protein with a resulting molecular mass of 78 kDa [14]. In contrast, other mammalian forms of calcineurin are highly conserved compared to the bovine enzyme. Sequence comparison using the BLAST algorithm (SIB BLAST Network Service; http://ca.expasy.org/cgi-bin/blast.pl) showed 99% sequence identity for the catalytic subunit from different species (bovine, human, mouse, and rat) The structural differences between the *Dictyostelium* enzyme and the enzyme from higher eukaryotes (bovine, human, mouse, and rat) is the likely explanation for the different observed responses to polyunsaturated fatty acids. The activity of other calmodulin-dependent enzymes has been reported to be influenced by unsaturated fatty acids with inhibition and activation observed with different target enzymes [15–18]. For some target enzymes, a bimodal effect was reported [16] with activation at low concentrations of fatty acids and inhibition at higher concentrations.

3.4. Inhibition by leukotriene B4 isomers and lipoxins

The inhibition by polyunsaturated fatty acids did not seem to match the pattern found with tyrphostins and retinoids, likely because of the absence of conjugated double bonds. Leukotrienes are another class of lipid molecules with three conjugated double bonds. Leukotriene B4 and select isomers were tested as possible inhibitors and all were found to inhibit albeit many weakly (Fig. 4). The related compounds, hydroxy-eicosatetraenoic acids, were not tested to limit the possible involvement of chain substituents on the possible effects on calcineurin. Unlike the polyunsaturated fatty acids, the inhibition by leukotrienes was not blocked by calmodulin. In this regard, the leukotrienes were similar to the tyrphostins and retinoids. The inhibition observed with calmodulin showed greater statistical significance according to analysis using Student's t test (not shown). One isomer, 6-*trans*-12-epi-leukotriene B4 (57% inhibition at 20 μM with $p < 0.000002$) was more effective than the other leukotrienes and was characterized further. With calmodulin included, the IC_{50} was determined to be 16.4 μM, a value similar to the IC_{50} value for tyrphostin A8 [7].

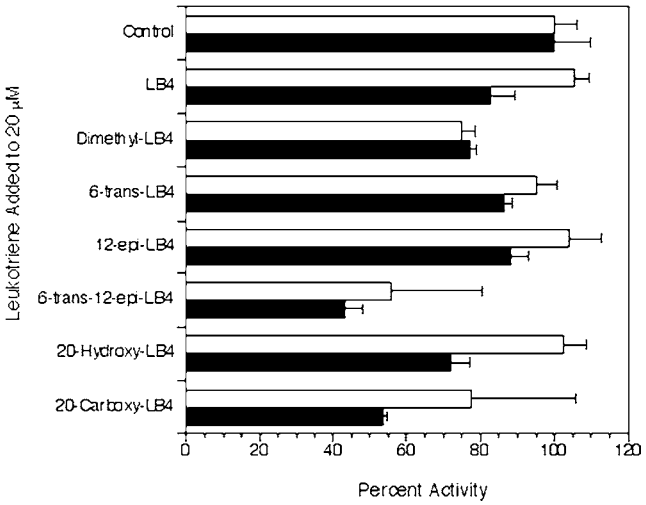

Fig. 4. Inhibition by leukotriene B4 isomers. Shown is a comparison for the inhibition of calcineurin by the different isomers of leukotriene B4 in the absence (open bars) and in the presence of 5 μg/ml calmodulin (filled bars). Activity was measured using *p*NPP as the substrate. Each bar represents the average of three measurements with the error bar showing the standard deviation of the measurements.

Lipoxins A4 and B4 also have conjugated double bonds. These compounds have four double bonds conjugated compared to the three double bonds found in the leukotrienes. Both lipoxins inhibited calcineurin and there was no apparent protection by calmodulin. Lipoxin B4 was a more effective inhibitor than lipoxin A4 (Fig. 5). The IC_{50} for lipoxin B4 was approximately 42 μM with pNPP as the substrate, close to the value for retinoic acid.

3.5. Significance of inhibition by lipids

Any potential biological significance of these findings is unclear. Certainly the concentrations used for effective inhibition of calcineurin were higher than the levels typically found in organisms. Nonetheless, there are numerous tantalizing reports in the literature that relate to possible connections between calcineurin and the various lipid molecules tested as inhibitors.

Calcineurin and fatty acids both have been implicated in cardiac physiology, although no direct linkage has been reported. Calcineurin is a critical component of the signaling pathway resulting in a hypertrophic response following myocardial infarction. Deng et al. [19] showed that calcineurin activity was elevated 1.9-fold and was involved in hypertrophic remodeling following myocardial infarction. Polyunsaturated fatty acids have been reported [20,21] to block arrhythmia associated with infarction with one possible mechanism being the inhibition of calcineurin. There is an apparent relationship between fatty acid metabolism and cardiac hypertrophy [22,23], although no involvement with calcineurin activity has been identified. Fatty acids and calcineurin seem to induce opposing actions in cardiac functions [20–22,24–28]. Our findings that fatty acids can inhibit calcineurin in vitro are suggestive that this effect may be a possible mechanism for the linkage of calcineurin regulation to metabolism and its broader involvement in cardiac physiology.

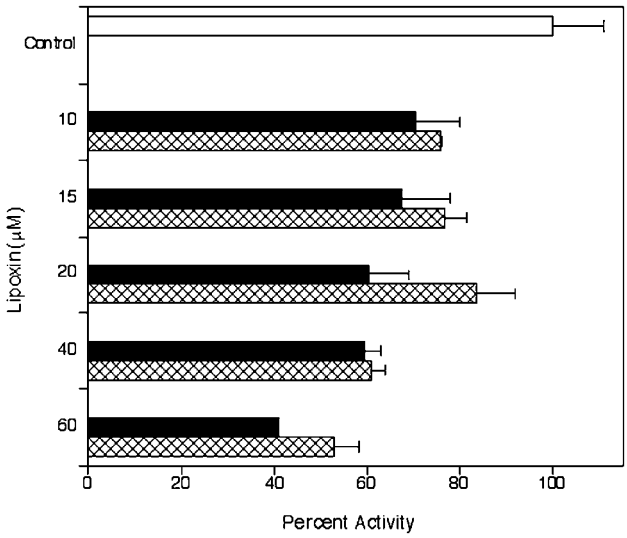

Fig. 5. Inhibition by lipoxin A4 and B4. Shown is a comparison for the inhibition of calcineurin by lipoxin A4 (hatched bars) and lipoxin B4 (filled bars) in the presence of 5 μg/ml calmodulin. Concentrations of LXA4 and LXB4 were varied as shown. The data were compared to activity in the absence of any lipoxin (open bar). Activity was measured with *p*NPP as substrate. Each bar represents the average of three measurements with the error bar showing the standard deviation of the measurements.

Fatty acids have also been shown to have effects in other systems regulated by calcineurin, such as the expression of interleukin-2 (IL-2) in T-lymphocytes stimulated by calcineurin [29,30]. Eicosapentaenoic acid and docosahexaenoic acid have been shown to be antiproliferative agents toward lymphocytes [31,32] and cause the down regulation of genes in T-cells. Although not suppressing the expression of IL-2 in these cells, fatty acids were reported to down regulate the secretion of IL-2 [33,34] and the expression of the α-isoform of IL-2 receptor [35]. The use of calcineurin inhibitors was found to cause the same effect as arachidonic acid on metabotropic glutamate receptors studied in synaptosomes [36]. Inhibition by these fatty acids, though likely dependent on modulating the calmodulin activation of calcineurin, may be another general mechanism for regulating calcineurin function.

Leukotriene B4 has been reported to stimulate IL-2 production [37] and augment IL-2 mediated responses [38], a result consistent with the failure of leukotriene B4 to cause significant inhibition of calcineurin. The leukotriene most effective as an inhibitor, 6-*trans*-12-epi-leukotriene B4, does not have significant biological activity compared to its parent molecule. Leukotriene B4 and lipoxins were reported to have opposing effects on inflammation with leukotriene B4 being pro-inflammatory and the lipoxins having anti-inflammatory activity [39]. Limited evidence [31,40] suggest that leukotriene B4 and lipoxins do not share biological actions with polyunsaturated fatty acids, and may have opposing actions in select target tissues. For example, leukotriene B4 seems to have an opposite effect on cardiac hypertrophy than do polyunsaturated fatty acids [40]. These biomolecules, moreover, cause physiological responses through the action of receptor molecules that were not included in the assays. These observations demonstrate that it is difficult to ascribe any physiological function to the in vitro inhibition of calcineurin.

The data for calcineurin inhibition do, however, provide information about the ability of these compounds to directly interact with calcineurin. As such, the data do provide insight toward the development of possibly novel and specific calcineurin inhibitors.

3.6. Significance for the development of calcineurin inhibitors

Inhibition by the polyunsaturated fatty acids seemed to result from modulating the activation of calcineurin by calmodulin. Only the fatty acids did not cause significant inhibition with calmodulin included and only the fatty acids did not have conjugated double bonds, although they were polyunsaturated. The leukotrienes and lipoxins inhibited calcineurin independently of the presence of calmodulin in the assay. The physiological significance of inhibition of calcineurin by any of these molecules is unclear, but these results are consistent with the hypothesis that conjugated double bonds are important determinants of effective calcineurin inhibition and verify that an aromatic ring structure is not needed for inhibition [7,8]. The nature of the double bonds was another difference between the polyunsaturated fatty acids and other lipids tested. The fatty acids contained only *cis*-double bonds whereas the other lipids had both *cis*- and *trans*-double bonds. The most effective inhibitor, 6-*trans*, 12-epi-leukotriene B4, also has an additional *trans*-bond compared to leukotriene B4. Inasmuch as inhibition by either 6-*trans*-leukotriene B4 or 12-epi-leukotriene B4 was not very different from the parent molecule, it seems the orientation of the 6-*trans*,12-epi-leukotriene B4 must be critical for better interacting with calcineurin. The presence and orientation of conjugated double bonds seem to be the primary structural difference explaining why the tested inhibitors evidently targeted different regions of calcineurin.

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