4-(Fluoromethyl)phenyl Phosphate Acts as a Mechanism-based Inhibitor of Calcineurin*

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Calcineurin, also known as protein phosphatase 2B, is a Ca2+- and calmodulin-dependent protein phosphatase consisting of a 59-kDa catalytic subunit (calcineurin A) and a 19-kDa Ca2+- binding subunit (calcineurin B). The A subunit shows extensive homology with the family of serine/threonine protein phosphatases that also includes protein phosphatases 1 and 2 (1), whereas calcineurin B is a member of the family of Ca2+- binding proteins that includes calmodulin, troponin C, and parvalbumin and is presumed to have a regulatory function.

By use of the immunosuppressant drugs cyclosporin A and FK506, calcineurin has recently been identified as having a role in the T-cell receptor signal transduction pathway. These drugs bind to distinct intracellular receptors (2–4) and form a complex that binds to and inhibits the phosphatase activity of calcineurin (5–7). Inhibition of calcineurin in T-lymphocytes prevents the formation of an active transcription factor necessary for the production of the cytokine interleukin-2 (8, 9). The discovery of calcineurin inhibition by cyclosporin A and FK506 has heightened interest in the enzyme, with many labs focusing research on the details of drug-mediated inhibition and aspects related to the enzymatic mechanism. Currently nothing is known about the active site environment.

Recent studies (10, 11)1 have described a pair of structurally similar inhibitors of protein-tyrosine phosphatases that appear to act as mechanism-based inhibitors. These novel phosphatase inhibitors, 4-(fluoromethyl)phenyl phosphate (FMPP)2 and 4-(difluoromethyl)phenyl phosphate (DFPP), are substrates that undergo transformation after enzyme-catalyzed hydrolysis to yield a reactive intermediate, presumably a quinone methide, that inactivates the enzyme by forming a covalent bond to an active site residue. Because p-nitrophenol phosphate and tyrosine phosphate are substrates for calcineurin, the fluoromethylphenyl phosphates, as analogues, might be hydrolyzed by it as well.

In this study we present data that identify FMPP as a mechanism-based inhibitor of calcineurin. The inhibitor produced a time-dependent, first order loss of calcineurin activity that was saturable, with stoichiometric and specific labeling of calcineurin A, the catalytic subunit. Addition of an alternate substrate protected the enzyme from inactivation. Exogenous nucleophiles had no effect on inactivation, indicating that the inactivation event is inaccessible to solvent nucleophiles and was therefore occurring at the active site. In addition, FMPP-treated enzyme remained completely inactive for a period of several days, demonstrating that the inhibition is essentially irreversible. This report represents the first demonstration of a mechanism-based inhibitor for calcineurin.

EXPERIMENTAL PROCEDURES

Materials—pNPP, bovine serum albumin, Sephacryl S-300, DEAE-Sepharose CL-6B, hydrogen peroxide, and pyridine were purchased from Sigma. Calmodulin was purified from bovine brain (12). Cysteine was purchased from Life Technologies, Inc. Cellex-CM was purchased from Bio-Rad. Ultima Gold scintillant was purchased from Packard (Meriden, CT). Phenol was purchased from J. T. Baker, Inc. (Phillipsburg, N.J.). PM30 membranes were purchased from Amicon (Beverly, MA). The starting phenols for the synthesis of the phosphate esters in Table I (p-cresol, p-fluorophenol, p-iodo phenol, p-trifluoromethylphenol, p-cyanophenol, and p-bromophenol) were purchased from Aldrich.

Methods—Protein concentrations were measured using the Bio-Rad assay (13) with bovine serum albumin as a standard. The para-substituted phenylphosphate esters listed in Table I were synthesized as described (14). The sodium salt was prepared from the cyclohexylammonium salt by exchange over a Cellex-CM column (Na+ form). FMPP, [3H]FMPP, and AMPP were synthesized as described.

Purification of Recombinant Calcineurin—Recombinant calcineurin was purified as described (15). Briefly, crude extract of recombinant calcineurin A and recombinant calcineurin B purified through the first DEAE-Sepharose CL-6B chromatography step were combined and stirred on ice overnight. CaCl2 was then added to a final concentration of 6.0 mM. Reconstituted calcineurin (calcineurin A + calcineurin B) was purified using a calmodulin-Sepharose affinity step. Following chromatography, column fractions containing reconstituted calcineurin were combined and concentrated using an Amicon pressured filtration cell equipped with a PM30 membrane. The concentrated protein frac-

1 This work was supported by National Institutes of Health Grants GM46865 (to F. R.) and GM47018 (to T. S. W.), American Cancer Society Research Award JFRA-490 (to T. S. W.), and a Camille Dreyfus Teacher Scholar Award (to T. S. W.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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905 mm) equilibrated with 20.0 mM Tris-Cl, pH 7.5, 0.10 mM KCl, 1.0 mM magnesium acetate, 1.0 mM dithiothreitol, and 0.10 mM EGTA at a flow rate of 20 ml/h. Column fractions were assayed for phosphatase activity using pNPP as substrate as described below. The protein was concentrated over a PM 30 membrane and stored at −20 °C in the same buffer.

Assay of Calcineurin Activity Using pNPP—Phosphatase activity was measured at 30 °C using pNPP as the substrate in 25 mM MOPS, pH 7.5, 1.0 mM MnCl$_2$, 0.10 mM CaCl$_2$, 1 μM calmodulin, and 0.8 μM calcineurin. After incubation for 2–5 min at 30 °C, the reaction was initiated by the addition of pNPP to a final concentration of 10.0 mM. Activity was measured by following the increase in absorbance at 410 nm with time for 3 min using a Cary 1 UV/visible spectrophotometer. The temperatures of both the sample and reference cuvette were maintained using thermostable cell holders attached to a Lauda RM6 circulating water bath.

Calcineurin Assays with FMPP and AMP-P—A stock solution containing 50 mM MOPS, pH 7.5, 2 mM MnCl$_2$, 0.2 mM CaCl$_2$, 31 μM calmodulin, and varying concentrations of FMPP or AMP-P was pre-warmed to 30 °C, after which 3 μl were removed and combined with 2 μl of calcineurin (final concentration of calcineurin varied between 4 and 79 μM). After incubation at 30 °C for varying times, the enzyme/inhibitor mix was diluted to 500 μl with 25 mM MOPS, pH 7.5, 1 mM MnCl$_2$, 0.1 mM CaCl$_2$, and 10 mM pNPP (final concentrations), and the activity measured. It is assumed that enzyme inactivation occurring via active site-directed or mechanism-based inhibitors follows a first order process as described in Equation 1 (16):

$$\ln \frac{e}{e_0} = -k_{inact}t(1 + K_I[I])$$  

(Eq. 1)

where $e$ is the enzyme activity at time $t$, $e_0$ is the enzyme activity at time zero, $k_{inact}$ is the rate constant for inactivation, $K_I$ is the Michealis constant for the inhibitor, and $[I]$ is the inhibitor concentration. Plots of $\ln \frac{e}{e_0}$ versus time at different concentrations of inhibitor should yield a series of first order rate constants ($k_{inact}$) for inactivation according to Equation 2:

$$k_{inact} = \frac{-1}{K_I[I]}$$  

(Eq. 2)

A double-reciprocal plot (Equation 3) will give a nonzero intercept of $-1/k_{inact}$, if the effect is saturable in $[I]$, and a slope of $-1/K_I/k_{inact}$:

$$\frac{1}{k_{inact}} = \frac{-1}{K_I[I]} - \frac{1}{K_I[k_{inact}]} \frac{1}{[I]}$$  

(Eq. 3)

Stoichiometry of Labelling—To determine the number of moles of adduct covalently bound to enzyme in FMPP-inactivated calcineurin, 15.5 μg of enzyme (final concentration, 20 μM) were inactivated with 60 μM [H]$^+$FMPP (10.3 μCi/μmol) in a total volume of 10 μl as described above. The sample was then electrophoresed on a 13% SDS-polyacrylamide gel, and the proteins were visualized with Coomassie staining. The gel slices containing each resolved polypeptide were excised, and the acrylamide was dissolved with 500 μl of 30% peroxide at 60 °C for 3.5 h. Scintillant was added prior to counting, and the stoichiometry was determined by assuming 100% recovery of protein during electrophoresis.

Substrate Protection and Effect of Solvent Nucleophiles—The substrate protection assays were carried out essentially the same as the inhibition assays with the exception that PMPP was included in the reaction buffer at the concentrations indicated in the text. The effect of solvent nucleophiles on calcineurin inhibition by FMPP was determined using the assay described above with 50 mM FMPP and 62.5 mM cysteine, 62.5 mM KCl, or 82 mM NaSCN.

Irreversibility of Inactivation—To demonstrate irreversible inactivation of calcineurin, the enzyme (29.6 μM) was incubated with 60 μM FMPP in 25 mM MOPS, pH 7.5, 1 mM MnCl$_2$, 1 μM CaCl$_2$, and 21 μM calmodulin at 30 °C for 30 min. It was then diluted with 360 μl of buffer B (20 mM Tris-Cl, pH 7.5, 1 mM magnesium acetate, 0.1 mM EGTA, and 10 mM β-mercaptoethanol). The activity remaining after the 30 min incubation was measured by diluting 50 μl of the inactivated enzyme mixture to 500 μl with 25 mM MOPS, pH 7.5, 1 mM MnCl$_2$, 0.1 mM CaCl$_2$, and 10 mM pNPP (final concentrations) and assaying as described above. The inactivated enzyme was then placed in a Pierce System 500 Microdialyzer equipped with a M, 3000 cutoff dialysis membrane and dialyzed against buffer B at 4 °C. The buffer was changed six times with 60 ml aliquots during the dialysis. 50-μl aliquots of enzyme were removed at the indicated times and assayed as above. Control samples with 75 mM PMPP or 75 mM PMPP and 60 mM FMPP were prepared and assayed in an identical fashion.

Malachite Green Assay—The activity of calcineurin toward the

phenyl phosphate esters in Table 1 was determined by measuring phosphate release using the malachite green assay as described by Lanzer et al. (17). The only modification was that the Sterox was not used.

RESULTS

FMPP Is an Inhibitor of Calcineurin—FMPP (10) and a related compound, DFPP (11), have been used as mechanism-based inhibitors of protein-tyrosine phosphatases. Calcineurin also has activity toward aryl phosphate esters, suggesting that it may be inactivated by these compounds as well. When recombinant calcineurin was incubated with varying FMPP concentrations, the enzyme showed a rapid loss of activity with inhibitor concentrations of 2–40 μM. After 30 s, greater than 70% of enzyme activity was lost with inhibitor concentrations of ≥20 μM. The loss of activity was linear over the time scale measured. When calcineurin was incubated with AMPP at concentrations up to 80 μM, no significant inhibition was apparent. A plot of $\ln (e/e_0)$ versus time of incubation with inhibitor indicated that the loss of activity is first order. Replotting the data as a secondary reciprocal plot, according to Equation 3, yields a straight line with a nonzero intercept (Fig. 1), indicating that inhibition is saturable. Calcineurin has a $K_I$ for FMPP of 44.4 μM and a $k_{inact}$ of 8.82 min$^{-1}$. The value of $k_{inact}$ and $K_I$ are similar to the turnover numbers and Michealis constants obtained with native bovine brain calcineurin determined for other phenyl phosphate esters (Table I).

Substrate Protection—We next investigated whether the presence of substrate would protect the enzyme from inactivation. PMPP was used for the substrate protection assays due to its similarity in structure to FMPP. Exposure of calcineurin to
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PMPP, at concentrations of 57 or 100 mM, did not significantly affect its activity toward pNPP after dilution, whereas in the presence of either 40 or 57 mM FMPP alone the enzyme was completely inhibited (Fig. 2A). When FMPP and PMPP were added to the enzyme simultaneously, at either equimolar concentrations or with PMPP in excess, the enzyme remained fully active over a 60-min period (Fig. 2A). p-iodophenyl phosphate was also able to protect calcineurin from FMPP inactivation (data not shown). Protection by PMPP is concentration-dependent, as was demonstrated by experiments in which the PMPP concentration was varied between 0 and 50 mM while keeping the FMPP concentration constant at 40 mM (Fig. 2B).

Effect of Solvent Nucleophiles—The presence of 62.5 mM cysteine had little affect on calcineurin activity (Fig. 3, filled circles), whereas 50 mM FMPP reduced the activity by 95% within minutes (Fig. 3, triangles). In the presence of cysteine (62.5 mM), calcineurin was still inactivated by 50 mM PMPP (Fig. 3, squares). The presence of either KCN (62.5 mM) or NaSCN (82 mM) also failed to prevent calcineurin inactivation by FMPP (data not shown).

Irreversibility of Inactivation—Incubation of calcineurin (29.6 μM) with 60 mM FMPP for 30 min at 30°C led to a complete loss of activity. After incubation, the inactivated enzyme retained less than 5% of its activity when compared with a control enzyme reaction. FMPP-treated enzyme remained inactive even after extensive dialysis, with less than 5% activity remaining after dialysis at 4°C for 119 h (Fig. 4). A control reaction containing PMPP but not FMPP remained active throughout the same time course, whereas a second control reaction containing both PMPP and FMPP had a slight loss of activity initially but remained active throughout the course of the dialysis (Fig. 4).

Stoichiometry of Labeling—If FMPP is a mechanism-based inhibitor of calcineurin, inactivation should produce stoichiometric labeling of the active site. To determine the stoichiometry of labeling, calcineurin was inactivated with [3H]FMPP, and the protein mixture was resolved via SDS-polyacrylamide gel electrophoresis. As is shown in Table II, it was found that calcineurin A was preferentially labeled, whereas calcineurin B and calmodulin contained only minor amounts of [3H]. Thus, incubation of 15.5 μg (0.2 nmol) of calcineurin led to the recovery of 1.5 × 10^−3 μCi of [3H] in the catalytic subunit with a fractional stoichiometry of labeling of 0.75 mol of [3H] per mol of subunit. On the other hand, the fractional stoichiometries of labeling of calcineurin B and calmodulin were 0.15 and 0.14, respectively.

**DISCUSSION**

Mechanism-based inhibitors of protein phosphatases are relatively new compounds. Two examples of these that show promise for unraveling aspects regarding the catalytic mechanisms of protein phosphatases are FMPP (10) and DFPP (11). In order to show that a compound inhibits via mechanism-based inhibition, a number of criteria need to be satisfied. These include a time-dependent, first order loss of activity; the observance of saturable inhibition kinetics; substrate protection; the lack of effect of solvent nucleophiles on the rate of inactivation; the covalent and stoichiometric attachment of the inhibitor to the enzyme; and the irreversibility of inactivation (18). Inhibition of calcineurin by FMPP meets the above criteria, indicating that inactivation is occurring at the active site via enzyme-mediated generation of a reactive species.

Inactivation of calcineurin by FMPP is rapid, with greater than 70% inactivation occurring within the first 30 s of incubation with FMPP at concentrations of 20 mM or higher. In all cases the inactivation was first order with a rate of inactivation proportional to the amount of inhibitor. A nonzero intercept on the ordinate of a double-reciprocal plot indicated saturable inhibition occurring at a rate of 8.82 min^−1. This rate constant is likely to be comprised of several rate constants, including those representing hydrolysis of the phosphate ester as well as any other chemical or enzymatic steps leading to a covalent enzyme adduct. This value is comparable with k_cat values of 7–35 min^−1 for ary1 phosphate esters substituted at the para position (Table I) and indicates that the rate-limiting step for inactivation of calcineurin by FMPP is likely to be hydrolysis of the phosphate ester.

The stoichiometry of inactivation was determined using [3H]FMPP. In these experiments, inactivation with [3H]FMPP...
led to near stoichiometric labeling of calcineurin, with the majority of the label incorporated into the catalytic subunit. Some label was also found associated with calcineurin B and calmodulin. Although this could represent nonspecific labeling of these proteins, it could also indicate that they are close enough to the active site to allow labeling by inhibitor diffusing out of the active site. Preliminary mass spectrometry experiments have indicated that FMPP-inactivated calcineurin A has a mass approximately 115 daltons larger than untreated enzyme (expected mass change is 106 daltons), whereas the mass of calcineurin B, as well as that of calmodulin, is unchanged (data not shown).

If FMPP inactivates in a mechanism-based fashion, an alternate substrate should compete with the inhibitor for access to the active site and protect calcineurin from inactivation, with the rate of inactivation inversely proportional to the substrate concentration. PMPP is identical in structure to FMPP, with the exception of an isosteric substitution of a hydrogen atom by fluorine. When FMPP was incubated with calcineurin at varying concentrations of PMPP, the enzyme was protected from inactivation, and this protection was dependent on the concentration of PMPP (Fig. 2B). A concentration of 1 mM PMPP provided little protection against 40 mM FMPP, 10 mM PMPP protected up to 60% of the activity over a period of 20 min, and 50 mM PMPP provided almost complete protection during the time course of the assay.

Even though inactivation of calcineurin occurs in a time-dependent fashion and substrate protects against inactivation, it does not prove that FMPP is a mechanism-based inhibitor. One other possibility is that the inhibitor forms a reactive species in solution, independent of catalysis, that is preferentially directed toward the active site. Another possibility is that enzyme activity is required to form a product that upon release into solution is able to form a reactive species that indiscriminately inactivates the enzyme. One way to distinguish between these two cases and one in which an electrophilic species is formed and reacts at the active site is to include an excess of a scavenger nucleophile in the reaction buffer. Any reactive species either formed in solution or released from the enzyme will react with this nucleophile and be quenched before it can reassociate with the enzyme in a nonspecific manner. When either cysteine, KCN, or NaSCN were present in the assay,
Calcineurin was still inhibited by FMPP, indicating that the reactive species was quenched from solvent, presumably at the active site. Additional evidence for sequestration of the reactive species at the active site is provided by experiments with AMP (Fig. 5, 4). AMP can be hydrolyzed by calcineurin, but because acetate is a poor leaving group, elimination of acetate is slower than that of fluoride. The initial hydrolysis product can then be released into solution before a reactive quinone methide is formed. AMP had little inhibitory effect on calcineurin, indicating that if a reactive species forms from this substrate, it quickly becomes deactivated by solvent.

If FMPP is inactivating calcineurin by forming a chemically stable adduct with an active site residue, the inactivation should be irreversible. Extensive dialysis (119 h) with several changes of buffer did not reverse inactivation of calcineurin by FMPP. To show that prolonged dialysis was not incompatible with recovery of activity, a sample of calcineurin dialyzed in parallel with buffer from the presence of FMPP instead of FMPP remained active. This sample showed a gradual loss of activity with time, with greater than 70% activity remaining after 119 h. A second control sample, with both PMPP and FMPP present, showed an initial drop in activity, as expected due to the presence of FMPP, but at 17 h of dialysis its activity paralleled that for the uninactivated enzyme (Fig. 4).

In the case of inactivation by both FMPP and DFPP, it is believed that the enzyme catalyzes phosphate ester hydrolysis, elimination of fluoride occurs, generating a quinone methide (10). This reactive electrophile can then be attacked by a nearby nucleophile, resulting in a covalent modification (Fig. 6). If fluoride elimination is rapid it may occur before release of product from the active site, resulting in covalent modification of an active site residue. The data presented indicate that FMPP is indeed a mechanism-based inhibitor of calcineurin.

As a reagent that inactivates by reaction with active site residues, FMPP will be useful for identifying calcineurin residues that participate in substrate binding and/or catalysis; experiments with this aim in mind are currently in progress. At present, there is nothing known about the active site of calcineurin or other enzymes in the serine/threonine protein phosphatase family such as protein phosphatases 1 and 2A. Given the fact that these members share extensive homology, it is likely that they catalyze phosphate ester hydrolysis in a mechanistically similar fashion. A comparison of the primary sequences of calcineurin with protein phosphatases 1 and 2A indicates an active site domain of ~200 residues and within this domain, six regions of very high conservation consisting of approximately 60 amino acids that probably represent either active site residues or structurally important regions that have been conserved during evolution (19–21).

Since the discovery of its involvement in T-cell activation, calcineurin has become the focus of a number of studies aimed at determining its structure and function. Certainly one of the aims of these studies is to design novel calcineurin inhibitors that retain immunosuppressive activity but lack the toxic side effects noted for these powerful transplantation drugs. With a Ki for inactivation in the millimolar range, FMPP is not an ideal inhibitor for calcineurin. However, the value of 44.4 mKm does compare with Ki values for substrates with analogous structures (Table I). The fact that certain phosphopeptide and phosphoprotein substrates of calcineurin have Ki values in the micromolar range (22–24) indicates that additional structural elements could be utilized and, using FMPP as a model, incorporated to provide increased binding affinity for novel, potent, and specific calcineurin inhibitors.

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