Inhibition of the Calcineurin-NFAT Interaction by Small Organic Molecules Reflects Binding at an Allosteric Site

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Transcriptional signalling from the Ca^{2+}-calmodulin-activated phosphatase calcineurin to its substrate NFAT (also termed NFATc) is critically dependent on a protein-protein docking interaction between calcineurin and the PxIxIT motif in NFAT. Several inhibitors of NFAT-calcineurin association (INCA compounds) prevent binding of NFAT or the peptide ligand PVIVIT to calcineurin. Here we show that the binding site on calcineurin for INCA1, INCA2, and INCA6 is centered on cysteine-266 of calcineurin Aα and does not coincide with the core PxIxIT-binding site. Although ample evidence indicates that INCA1 and INCA2 react covalently with cysteine-266, covalent derivatization alone is not sufficient for maximal inhibition of the calcineurin-PVIVIT interaction, since the maleimide INCA12 reacts with the same site and produces only very modest inhibition. Thus inhibition arises through an allosteric change affecting the PxIxIT-docking site, which may be assisted by covalent binding, but which depends on other specific features of the ligand. The spatial arrangement of the binding sites for PVIVIT and INCA makes it probable that the change in conformation involves the β11-β12 loop of calcineurin. The finding that an allosteric site controls NFAT binding opens new alternatives for inhibition of calcineurin-NFAT signalling.

Ca^{2+}-calcineurin signalling serves vital purposes in mammalian cells, among them the provision of a direct link between mobilization of cytoplasmic Ca^{2+} and gene expression (1,2). In the physiological activation of T cells, calcineurin acts through NFAT-family transcription factors and other transcriptional effectors (3-6). Levels of calcineurin in T cells are limiting, and upward or downward modulation of calcineurin enzymatic activity is directly reflected in increased or decreased transcription from cytokine gene promoters (7-14). The immunosuppressive drugs cyclosporin A (CsA)¹ and FK506 owe their clinical effectiveness to the ability of CsA-cyclophilin or FK506-FKBP12 complexes to inhibit calcineurin in cells of the immune system (15,16). However, the beneficial actions of CsA and FK506 are counterbalanced by serious toxicities attributed at least in part to their interference with calcineurin signalling in other cells and tissues (17,18).

The centrality of calcineurin and the calcineurin-NFAT pathway to immune responses has suggested that interrupting signalling at any of several points could provide a therapeutic alternative to current immunosuppressive drugs. Promising target points are the Ca^{2+} signal that activates calcineurin (19,20), the protein-protein interaction of calcineurin and NFAT (21-25), and the cooperative binding of NFAT and AP1 on DNA (26-30). Each strategy has the potential to be more selective, and hence less toxic, than treatment with CsA or FK506, but none has yet advanced to the stage of small nonpeptide inhibitors that can be tested in vivo.

There is considerable evidence that targeting the calcineurin-NFAT protein-protein interaction will produce a selective inhibition. We have demonstrated that the calcineurin-NFAT interaction is based on recognition of a PxIxIT motif in NFAT, and that this recognition is essential for efficient signalling (23). Peptides that compete for binding at the PxIxIT-recognition site both inhibit the calcineurin-NFAT interaction in vitro and selectively inhibit calcineurin-NFAT signalling in cells (23,24,31). A recently published study shows that a competitor peptide modified to promote its uptake into cells can prevent heterologous graft rejection in mice (32). Further, high-throughput screening of a library of organic compounds has led to identification of nonpeptide inhibitors of NFAT-calcineurin association (INCA compounds) (25).
These compounds interfere with calcineurin-NFAT signalling in cells, motivating a continued search for inhibitors having higher affinity and reduced nonspecific toxicity, and suitable for in vivo administration to animals.

The further development of inhibitors can be guided by structural information about the sites of protein-protein interaction and inhibitor binding. To this end, we have determined the structure of the NFAT docking site on calcineurin, in which the PxIxIT recognition peptide of NFAT binds in an extended configuration and each conserved residue of the peptide directly contacts calcineurin (33). Here we extend the structural studies by identifying a distinct binding site for INCA compounds, at a cysteine residue adjacent to the PxIxIT peptide docking site. It is covalent binding or tight noncovalent binding of the bulky INCA compounds at this second site that allosterically inhibits recognition of PxIxIT peptide and NFAT. Our findings serve to identify the approaches that are most likely to be fruitful in developing improved inhibitors. In addition they suggest that calcineurin-substrate recognition, like calcineurin catalytic activity (34-37), may be modulated physiologically by redox reactions.

EXPERIMENTAL PROCEDURES

cDNA constructs, protein expression, and synthetic peptides— The expression construct for GST-calcineurin(2-347), encoding GST and the catalytic domain of human calcineurin Aα, calcineurin(2-347), encoding GST and the catalytic domain of human calcineurin Aα with the substitutions Y341S, L343A, and M347D, has been described (24). Constructs encoding mutant proteins with individual C>A or C>V replacements, or with the combined C>A/C266 and C>A/V266 replacements, were made by PCR mutagenesis with appropriate primers and subcloning. In each case the sequence of the cDNA insert was verified.

GST-calcineurin fusion proteins were expressed in E. coli strain BL21-CodonPlus-RP (Stratagene) by overnight growth at 18°C after addition of 1 mM IPTG. Calcineurin was purified from the bacterial lysate by affinity chromatography on Glutathione Sepharose (Amersham Biosciences) and cleavage in 50 mM Tris·HCl pH 7.0, 150 mM NaCl, 1 mM EDTA, 1 mM DTT with PreScission Protease (Amersham Biosciences). Purified calcineurin(2-347) was concentrated by Centricon-30 filtration, aliquotted, flash frozen, and stored at −80°C. In most cases, the concentration of DTT in the buffer was reduced to 0.1 mM during concentration of the protein. This step and the further substantial dilution into fluorescence polarization assays minimized any interference of residual DTT with the INCA compounds. In some cases, protein was concentrated in buffer free of DTT immediately before freezing.

PVIVIT 14-mer peptide (24) was labelled overnight at room temperature in a reaction containing 2 mg peptide, 1.5 mg Oregon Green (Oregon Green 488 carboxylic acid, succinimidyl ester *5-isomer*; Molecular Probes), and 5 µl diisopropylethylamine in 190 µl anhydrous N,N-dimethylformamide. The labelled peptide was purified by C18 reversed-phase HPLC. The calcineurin(254-273)/C256S peptide, RGSSYFSYPACVEFLQHNN, was synthesized and HPLC purified at Tufts University Core Facility, and stored desiccated at −20°C.

Inhibitors— INCA1, INCA2, INCA6, and INCA12 (Figure 1) were obtained from ChemBridge. Inhibitor stocks were prepared at 10 mM in anhydrous DMSO and stored desiccated at −20°C. Corresponding aliquots of anhydrous DMSO were stored desiccated at −20°C for addition to control incubations. DMSO at concentrations up to 1% had no effect on the parameters studied.

Fluorescence measurements— Fluorescence measurements were made on 10 µl samples in a black 384-well plate (Molecular Devices) using the fluorescein filter set (excitation 485 nm, emission 530 nm) in an Analyst plate reader (Molecular Devices). Each final reaction in 100 mM NaCl, 2 mM Mg acetate, 20 mM HEPES pH 7.4, 0.1% (w/v) bovine IgG contained calcineurin, 100 nM fluorescent PVIVIT, and other additions specified. Calcineurin was omitted for measurements of the fluorescence emitted by unbound peptide. In competitive binding assays, calcineurin was typically present at 1 µM or 1.5 µM; in direct binding titrations, calcineurin was used at 0.15 µM to 10 µM. Except in time course experiments, adequate time was allowed for the signal to reach a stable value, as verified by repeated readings of the same samples. Pretreatment with IAM, NEM, or INCA12 was for 30 min to 2 hr.

Data from direct titrations with calcineurin have been fitted to a model for equilibrium binding to a single class of sites (38). Competition and time course data for INCA compounds have been plotted without attempting to fit a theoretical curve, since the data are not sufficient to specify parameters for covalent binding, noncovalent binding, and side reactions of the INCA compounds.

The stability of calcineurin-INCA2 association was examined after equilibration of 6 µM calcineurin, 100 nM labelled PVIVIT, and 20 µM INCA2 to produce an essentially complete blockade of PVIVIT binding. To assess short-term stability of the complex, samples were extracted by vortex mixing with 2 volumes of buffer-saturated ether for 2 min, and briefly centrifuged to separate the phases. Extraction of samples containing only calcineurin and labelled PVIVIT had no effect on peptide binding and did not prevent subsequent
displacement of peptide by INCA2. Extraction of samples containing only INCA compound, prior to incubation with calcineurin and PVIVIT, demonstrated that removal of unbound INCA2 from the aqueous phase was complete within 10 sec. To assess long-term stability of the complex, samples containing PVIVIT and blocked calcineurin were supplemented with 5 mM NEM or 5 mM DTT, and PVIVIT binding was monitored during the following 4 hr. Because the calcineurin concentration in fluorescence polarization assays is greater than the $K_d$ of the calcineurin-PVIVIT interaction, each of these procedures is a sensitive test for unmasking of blocked PVIVIT-binding sites.

**Synthetic peptide-INCA reactions**— Freshly dissolved RGSSYFYSYPAVCEFILQHNN peptide, 6 $\mu$M in 200 mM NaCl, 4 mM Mg acetate, 20 mM HEPES pH 7.35, in 20 $\mu$l total volume, was incubated for 60 min at room temperature with 50 $\mu$M INCA1, INCA2, or INCA6. At the end of this incubation, samples were either immediately diluted into 240 $\mu$l 0.1% (v/v) TFA and frozen; or further incubated for 60 min after addition of NEM to 1 mM, then diluted into 0.1% (v/v) TFA and frozen. Control samples were reacted first with 1 mM NEM for 60 min, then with 50 $\mu$M INCA compound for 60 min.

**Mass spectrometry**— For MALDI-TOF mass spectrometry, samples were desalted and concentrated by binding the peptides to C18 resin in a ZipTip (Millipore) and eluting with 70% (v/v) acetonitrile, 0.1% (v/v) TFA. Eluted peptides were diluted 1:10 with $\alpha$-cyano-4-hydroxycinnamic acid matrix (10 mg/ml in 50% (v/v) acetonitrile, 0.1% (v/v) TFA) and dried onto the target plate. Mass spectra were obtained using an Applied Biosystems Voyager-DE STR instrument operated in reflector mode, with accelerating voltage 20 kV, grid voltage 72%, guide wire 0.1%, and extraction delay time 175 ns. Calibration was performed using the MASCAL2 mass standard set (Sigma).

A minor peak frequently observed in the peptide sample, having mass $\sim$1776 Da, is apparently the peptide fragment RGSSYFYSYPAVCEF. Corresponding peaks in samples that had been incubated with INCA1, INCA2, or NEM—or in samples subjected to prolonged incubation with INCA6 to allow the reaction to approach completion—were shifted by the same mass as the main peak in those incubations. A peak of mass $\sim$1397 Da, matching the calculated mass of the fragment RGSSYFYSYPAV, was present occasionally both in untreated peptide samples and in samples that had been incubated with NEM or INCA compounds. The reaction, or lack of reaction, observed with these peptide fragments reinforces the conclusion that all the compounds investigated reacted with the cysteine –SH group.

**Cellular assays**— Dephosphorylation of NFAT, nuclear import of NFAT, and induction of cytokine mRNAs in D5 T cells were assessed as described in previous publications (23-25). Calcineurin activity in cell lysates was measured using a standard assay (39) with phosphorylated RII peptide as substrate. D5 T cells were chilled on ice, collected by centrifugation at 4°C, and resuspended in 50 mM Tris HCl pH 7.5, 1 mM EDTA, 0.1 mM EGTA, 0.2% (v/v) NP-40, 50 $\mu$g/ml PMSF, 10 $\mu$g/ml leupeptin, 10 $\mu$g/ml aprotinin, and where specified 30 mM sodium pyrophosphate. After 5 min on ice, lysates were centrifuged, and supernatants were transferred to fresh tubes containing an equal volume of 50 mM Tris-HCl pH 7.5, 100 mM NaCl, 2.5 mM CaCl$_2$, 100 $\mu$g/ml BSA, and 1.5 $\mu$m okadaic acid. Blank samples were prepared with resuspension buffer in place of cell lysate. The reaction was initiated by addition of $^{32}$p-labelled RII peptide, each tube was incubated for 15 min at 30°C, and the reaction was terminated by the addition of 100 mM potassium phosphate buffer containing 5% (v/v) trichloroacetic acid. Each sample was applied to a Dowex AG 50W-8X cation exchange column (Bio-Rad), and the effluent was collected and its content of radiolabel determined by liquid scintillation counting.

**Structural modelling**— Structural modelling was based on the coordinates of calcineurin Aca from PDB entries 1AU1 and 1TCO (40,41). The coordinates of docked PVIVIT peptide were from Li et al. (33). Figure 9 was prepared using RasMol.

**RESULTS**

The compounds INCA1, INCA2, INCA6, and INCA12 (Figure 1) have been shown to inhibit recognition of NFAT by calcineurin, assessed quantitatively as binding of fluorescent PVIVIT peptide to calcineurin. Several observations raised the possibility that a covalent INCA-protein complex is involved in inhibition of PVIVIT peptide binding. First, the most effective inhibitors were quinones or quinoneamines, compounds that are chemically reactive and that are known to form protein adducts. Second, the kinetics of binding were slow at intermediate concentrations of INCA compound, as illustrated for INCA1 (Figure 2A), with a plateau level of inhibition reached only after tens of minutes at room temperature. This behavior is often indicative of formation of a covalent ligand-protein complex. Finally, INCA1, INCA2, and INCA6 were inactivated as inhibitors in the competitive binding assay by preincubation with DTT (Figure 2B and not shown). Inactivation could reflect either reduction of the compounds by DTT to a less reactive form or covalent reaction with DTT.
Effect of sulfhydryl-modifying reagents— Protein sulfhydryl groups are likely sites of covalent modification by quinones. Consistent with this possibility, preincubation of calcineurin with the sulfhydryl-modifying reagent iodoacetamide (IAM) impaired INCA2 competition, whereas preincubation of calcineurin by itself had no effect (Figure 3A). In more extensive studies, preincubation of calcineurin with the maleimide INCA12 impaired competition by INCA1, INCA2, and INCA6 (Figure 3B and not shown).

Pretreatment of calcineurin with the sulfhydryl-modifying reagents IAM, N-ethylmaleimide (NEM), and INCA12 reduced the calcineurin-PVIVIT polarization signal even in the absence of INCA competitor (Figure 3 and not shown). Very low concentrations of INCA12 were sufficient to reduce the calcineurin-PVIVIT polarization signal, but even high concentrations did not fully eliminate the signal from bound PVIVIT (Figure 4A). Similarly, for high concentrations of IAM and NEM, the calcineurin-PVIVIT signal settled to a plateau that was well above the signal of free peptide (not shown). The explanation for this behavior, documented below, is that pretreated calcineurin retains the ability to bind PVIVIT with reduced affinity.

In principle, the effect of the nonspecific alkylating reagents IAM and NEM could be due either to true impairment of calcineurin-peptide binding or to impaired immobilization of the fluorescent label when PVIVIT peptide is bound. The second case would be evident in a reduction in the maximal polarization signal obtained by titrating fluorescent PVIVIT with IAM-pretreated or NEM-pretreated calcineurin, and a corresponding reduction in the slope of the central portion of the binding curve. In fact the curves for pretreated calcineurin showed no change in slope, but instead were shifted rightward to an extent compatible with a 1.5-2 fold loss in affinity of the calcineurin-peptide interaction (Figure 4B). Thus IAM and NEM are themselves partial inhibitors of the calcineurin-PVIVIT interaction, and experiments described below indicate that they act at the same site as the INCA compounds.

Identification of a target cysteine residue— The inhibitory activity common to IAM, maleimides, and quinones/quinoneimines strongly supported the notion that these compounds derivatize a cysteine sulfhydryl of calcineurin. Pilot experiments examining the effect of several single C>A substitutions in calcineurin on the inhibitory activity of INCA2 pointed to C266 as the likely reactive residue, and more detailed study confirmed that the point mutant C266A was insensitive to the inhibitory effect of INCA2 (Figure 5). Similar experiments demonstrated that this substitution also compromised the effectiveness of INCA1, INCA6, IAM, and maleimides (not shown).

Conversely, the other surface-exposed cysteine sulfhydryl groups were not required for the action of INCA compounds. The crystal structure of calcineurin shows six exposed cysteine residues in the catalytic domain, C166, C184, C228, C256, C266, and C336. We compared calcineurin in which all exposed cysteine side chains except C266 were changed to alanine (C>A//C266) and calcineurin with the same substitutions plus a C266V substitution (C>A/V266). Binding of fluorescent PVIVIT to the two proteins was comparable (Figure 6A). However, while replacement of the cysteines other than C266 did not compromise the effectiveness of INCA1, INCA2, or INCA6, the C266V substitution in the context of these other C>A replacements completely blocked the effects of the inhibitors (Figure 6B and not shown). The C266V substitution in this context also blocked the effect of NEM (not shown). These observations showed that the presence of C266 is necessary and sufficient for a complete block of PVIVIT binding by INCA compounds.

Formation of a peptide-INCA adduct— We tested the ability of the three INCA compounds to react with the synthetic calcineurin peptide RGSSYFYSYPAYRVCEFLQHNN. The peptide is calcineurin(254-273), with a C256S substitution to eliminate the possibility of covalent reaction at that position.

MALDI-TOF mass spectrometry demonstrated that INCA1 and INCA2, at micromolar concentrations, react covalently with the synthetic calcineurin peptide (Figure 7A-C). Peptide-INCA adduct formation was blocked in each case by prior incubation with excess NEM under conditions that derivatized the –SH group (Figure 7A and not shown). Conversely, NEM failed to react with the peptide-INCA adducts present after a first incubation with INCA1 and INCA2 (Figures 7B and 7C), confirming that the INCA compounds block the sulfhydryl group. Since the peptide is unlikely to have a preferred conformation in solution, the experiments show that INCA1 and INCA2 are sufficiently reactive to modify accessible cysteine sulfhydryl groups without necessarily forming an initial noncovalent complex.

INCA6 reacted more slowly (Figure 7D), with full labelling of the peptide requiring several hours. Reaction was again blocked by pretreatment with excess NEM. The sluggish reaction suggests that covalent reaction with C266 in calcineurin would require higher concentrations of INCA6, a local environment that increases the nucleophilicity of the C266 thiol, or a noncovalent interaction that assists in targeting INCA6 to the site.

Direct examination of tryptic digests of INCA6-treated calcineurin did not provide evidence of an INCA-protein adduct. Rather, the same tryptic peptide containing C266— identified by comparison with
digests of C266V calcineurin— was detected in the digests of untreated and treated calcineurin. However, it has been difficult to demonstrate adducts in tryptic digests of other proteins that are known to be covalently modified by quinones, probably because the S-quinone bond is labile (42, 43). Lability of the adduct would be less likely to prevent its detection in the experiments with synthetic peptide, where INCA compound is present in excess and the initial reaction mixture is directly examined by mass spectrometry.

**Reversibility of modification by INCA2**— Physical association of INCA compounds with calcineurin has been demonstrated by NMR spectroscopy and by cochromatography (25). Two further results indicate that spontaneous dissociation of INCA2, if it occurs, is extremely slow. First, extraction with ether for 2 min did not restore PVIVIT binding after blockade with the INCA compound was complete, even though the nonpolar INCA2, when not bound to calcineurin, rapidly partitions into ether. Second, incubation of INCA2-blocked calcineurin with excess NEM, at an NEM concentration that rapidly blocks the inhibitory site on unmodified calcineurin, did not lead to any recovery of PVIVIT binding during the subsequent 4 hours. The modification by INCA2 is nevertheless chemically labile, since treatment with excess DTT largely reversed the inhibitory effect.

**Effects of INCA2 in cells**— The chemical reactivity of INCA1 and INCA2 has raised the issue of their suitability for cellular studies. In previous work, INCA6 inhibited the dephosphorylation of NFAT and calcineurin-NFAT signalling, without inhibiting calcineurin enzymatic activity (25). INCA1 and INCA2 were not studied at that time because of their cytotoxicity, and even the less reactive INCA6 exhibited toxicity in some types of cells.

It proved possible to examine the effects of INCA2 in D5 T cells, for which INCA2 is not cytotoxic when used at low micromolar concentrations. At first glance, INCA2 had effects similar to those of INCA6, preventing the dephosphorylation of NFAT, the nuclear import of NFAT, and the induction of mRNAs encoding tumor necrosis factor-α (TNFα), interferon-γ (IFNγ), and macrophage inflammatory proteins MIP-1α and MIP-1β (Figure 8A-C, and not shown). However, closer examination revealed that the physiological effects were associated with a general inhibition of calcineurin catalytic activity (Figure 8D). A likely mechanism is oxidation of calcineurin, through reactions involving INCA2 itself or involving reactive oxygen species derived from INCA2 metabolism, effects that are probably exacerbated by INCA2 partitioning into lipids to produce elevated local concentrations of the quinone in cells. These new data reinforce the point (25) that current INCA compounds are most suited to probing the calcineurin-NFAT interaction in vitro, and that their use to inhibit the calcineurin-NFAT pathway in cells requires stringent controls.

**DISCUSSION**

The principal result of this study has been to locate the binding site on calcineurin for the calcineurin-NFAT signalling inhibitors INCA1, INCA2, and INCA6. The position of the INCA-binding site, in the vicinity of C266 of calcineurin, and its spatial relationship to the previously mapped NFAT docking site on calcineurin are illustrated in Figures 9A and 9B. The distance from S' of C266 to the nearest proline ring atom of the bound PVIVIT peptide is greater than 15Å. Thus, contrary to expectation, the most efficacious INCA inhibitors do not displace PVIVIT peptide and NFAT by binding competitively in the core PxIxIT recognition site, but rather they act by inducing an allosteric change in the NFAT-binding site.

The probable locus of the allosteric change is the β11-β12 loop of calcineurin, which positions residues F299 and P300 to form the proline and isoleucine pockets of the peptide-binding site (33). C266 and other residues in or just preceding helix 10 are in intimate contact with the residues that anchor both ends of the β11-β12 loop (Figure 9C): Y262 makes extensive contacts with the side chains of R292 and S301 and forms a template for the peptide backbone from residue 292 to residue 295; V265 and L269 are in contact with S301; and C266 itself is exposed in a canal on the surface of calcineurin, nestled against Y262 and in loose contact with the side chains of S294 and S301. Binding of a ligand in direct contact with the C266 sulphydryl, whether binding is covalent or noncovalent, is likely to require a local structural rearrangement and could alter the conformation of the β11-β12 loop. In addition to any changes in the immediate neighborhood of C266, movement of helix 10 could also reposition L275, in the short segment connecting helix 10 and β strand 10, thereby altering its packing against F299 and its contribution to formation of the proline pocket.

A concise explanation of the inhibitory effect of INCA compounds is that introduction of these substituents at C266 induces a structural rearrangement that alters the PVIVIT docking site, and that formation of a covalent —S-INCA bond provides part of the energy for the rearrangement. In fact, all the efficacious INCA compounds have the potential for covalent binding. INCA1 and INCA2, in particular, are highly reactive with the cysteine sulphydryl group. Their specific action on PVIVIT binding, via derivatization of C266, could be simply a byproduct of their general reactivity, or there could be a noncovalent interaction that targets the compounds to the vicinity of
C266 prior to covalent binding. INCA6 is less intrinsically reactive under our experimental conditions. Efficient formation of a calcineurin-INCA6 covalent adduct would require assistance, through targeting of INCA6 to the site or through heightened reactivity of the C266 sulfhydryl. The possibility also remains that INCA6 is not primarily a covalent inhibitor.

A more refined analysis of the reaction with calcineurin will entail characterization of the calcineurin-INCA adducts that arise under physiological conditions. Steric and other constraints in the protein might favor products that differ from those of the reaction with synthetic peptide discussed in the legend to Figure 7. For example, the INCA2 linkage to protein could involve a thioether bond, as observed in quinone cofactors of some amine dehydrogenases (44,45) and as inferred for many other protein-quinone adducts (42,43,46-49); or an ipso adduct at the imine carbon, as observed in the complex of N-acetyl-p-quinoneimine with papain (50). Cysteinyl-quinone thioether adducts are themselves highly reactive when in the oxidized form (51), and could undergo further reactions following initial adduct formation. An alternative mechanism of quinone-initiated modification of proteins is the production of reactive oxygen species (52), which in their turn react with cysteine side chains to give oxides of sulfur or sulfenyl amides (52,53). However, NMR measurements support a physical interaction between calcineurin and INCA compounds (25), and the experiments with synthetic peptide yielded no evidence that the INCA compounds oxidize cysteine sulfhydryl groups under our conditions.

A sidelight to our experiments is that the covalent inhibitors IAM, NEM, and INCA12 react with C266, and reduce the affinity of calcineurin for PVIVIT measurably, but much less dramatically than INCA1, INCA2, and INCA6. This finding highlights the role of an induced conformational change, rather than simple covalent derivatization of C266, in causing inhibition. It further serves as an indicator that other partial inhibitors of calcineurin-PVIVIT binding identified by high-throughput screening (25) may bind covalently or noncovalently at this allosteric site.

The current experiments were undertaken as a step toward the identification of better inhibitors of calcineurin-NFAT signalling. Our earlier mapping of the PxIXIT-binding site (33) has provided a structural template for the design of inhibitors that compete directly with the NFAT docking peptide. Inhibitors binding at the C266 site will present a less tractable task for structure-based design until the structure of the calcineurin-INCA complexes has been determined. Two immediately workable approaches suggested by our experiments are further high-throughput screening with modifications of the screening assay—such as use of C266V calcineurin or preincubation of library compounds with DTT or another thiol—that will tip the balance in the direction of noncovalent inhibitors; and examination of tethered ligands (54) focussed on the site we have defined. Less conventionally, it may prove possible to develop covalent ligands that are targeted to the site by a noncovalent interaction and are relatively unreactive with nonspecific sites (55,56). The pursuit of covalent inhibitors is an uncertain exercise in most cases, because of the difficulty of discriminating between a desired increase in the strength of the noncovalent interaction and an unwanted increase in chemical reactivity. Here, though, the combination of a single physiologically relevant site of interaction, judicious use of cysteine mutants, and careful control experiments may provide an avenue around this technical obstacle.

The sensitivity of protein-ligand affinity at the PxIXIT-binding site to minor modifications at C266, including carboxymethylation and the C266A substitution, raises an intriguing possibility for regulation of calcineurin signalling in cells. Previous work has focussed attention on the possibility that calcineurin catalytic activity is regulated by the physiological production of reactive oxygen species and oxidation of Fe(2+) at the active site (34-37,57-59). If redox reactions or endogenous regulators in cells also act at the INCA-binding site, they could complement redox modulation of overall calcineurin catalytic activity with a layer of regulation having selective effects on different substrates.

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REFERENCES


FOOTNOTES

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1 The abbreviations used are CsA, cyclosporin A; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; GST, glutathione S-transferase; IAM, iodoacetamide; IgG, immunoglobulin G; INCA, inhibitor of NFAT-calcineurin association; IPTG, isopropyl-β-D-1-thiogalactopyranoside; NEM, N-ethylmaleimide; PVIVIT, 14mer peptide MAGPHPVIVITGPHEE-amide; TFA, trifluoroacetic acid.
FIGURE LEGENDS

Figure 1. Structural formulae of the INCA compounds studied here. INCA1, INCA2, and INCA6 at micromolar concentrations completely block the calcineurin-PVIVIT interaction. INCA12 produces only partial inhibition of the calcineurin-peptide interaction.

Figure 2. A, Time course of inhibition of fluorescent PVIVIT binding by INCA1. INCA1 (13 µM) was incubated with calcineurin at room temperature or 0°C for the time indicated, the reaction was quenched by addition of 5 mM DTT and fluorescent PVIVIT, and the polarization of fluorescence was measured. The polarization signals from the calcineurin-PVIVIT mixture in the absence of INCA1 (bound) and from fluorescent PVIVIT alone (free) are indicated. A similar slow onset of inhibition was seen with intermediate concentrations of INCA2 and INCA6. B, Concentration dependence of PVIVIT displacement by INCA1 in the absence of DTT (filled symbols) and in the presence of 5 mM DTT (open symbols). Polarization signals from the calcineurin-peptide mixture in the absence of inhibitor and from free peptide are also shown. DTT had a comparable effect on displacement by INCA2 and by INCA6.

Figure 3. Effect of alkylating reagents on competition by INCA inhibitors. A, Concentration dependence of PVIVIT displacement by INCA2 from untreated calcineurin (filled symbols) and from calcineurin pretreated with 10 mM IAM (open symbols). B, Concentration dependence of PVIVIT displacement by INCA2 from untreated calcineurin (filled symbols) and from calcineurin pretreated with 30 µM INCA12 (open symbols). INCA12 likewise prevented displacement of PVIVIT by INCA1 and INCA6.

Figure 4. Effect of alkylating reagents on PVIVIT binding. A, Concentration dependence of PVIVIT displacement by INCA12. A prominent feature is that 1 µM INCA12, equimolar with calcineurin in the assay, substantially inhibits calcineurin-PVIVIT binding. Previous work (25) has shown that the plateau observed at high concentrations of INCA12 continues up to 1 mM INCA12. B, Titration of fluorescent PVIVIT with increasing concentrations of untreated calcineurin (black squares and left fitted curve), calcineurin pretreated with 10 mM IAM (grey squares and middle fitted curve), and calcineurin pretreated with 10 mM NEM (white squares and right fitted curve).

Figure 5. Competition of INCA2 with PVIVIT for binding to wildtype calcineurin (filled symbols) and the C266A mutant (open symbols). Note that binding to the mutant calcineurin in the absence of INCA compounds is lower than binding to wildtype calcineurin.

Figure 6. Competition by INCA compounds is strictly dependent on the presence of C266. A, Titration of fluorescent PVIVIT with increasing concentrations of C>A//C266 calcineurin (filled symbols) and C>A//V266 calcineurin (open symbols). B, INCA2 fails to inhibit PVIVIT binding to C>A//V266 calcineurin. The same result was obtained for INCA1 and INCA6. The difference in binding of PVIVIT to the two mutant proteins in the absence of competitor reflects the slightly lower affinity of C>A//V266 calcineurin for PVIVIT and possibly also a small mismatch in the protein concentrations in this experiment.

Figure 7. MALDI-TOF MS analysis of the reactions of NEM and INCA compounds with the synthetic calcineurin peptide. Data in each spectrum are plotted as intensity relative to the principal peak, and the abscissae have been labelled as mass (rather than as m/z) since all the species detected are singly charged. Values given are for the monoisotopic mass. A, The unmodified peptide (upper spectrum) is detected at 2382.02 Da (calculated mass, 2382.06 Da). There is neither disulfide formation nor oxidation of cysteine to a sulfenic acid, sulfenyl-amide, sulfinic acid, or sulfonic acid. A minor peak at 1775.62 Da is provisionally identified as the N-terminal fragment RGSSYFYSYPAVCEF (calculated mass, 1775.77 Da), as discussed in Experimental Procedures. After reaction with 1 mM NEM for 60 min (lower spectrum, displaced vertically for clarity) both the peptide and its N-terminal fragment have been
derivatized at a single site, which can be confidently identified as the cysteine sulfhydryl given the reaction pH, 7.35, and the presence of a free sulfhydryl group in the peptide sample. B-D, Reactions with INCA1, INCA2, and INCA6. The INCA compounds were present at 50 µM initially, and reaction was allowed to proceed for 60 min. The reaction step was followed in B and C by a further 60-min incubation with NEM; however, omission of the NEM incubation did not alter the products observed. The peptide-INCA adducts were not further characterized, but chemically reasonable product structures, based on the masses, are shown as insets. B, The INCA1 reaction yields a principal peak at ~2732 Da. Examination of this peak at higher resolution reveals a minor overlapping peak at ~2730 Da that we interpret as the oxidized form of the adduct (Supplementary Figure 1). The peak at ~2125 Da is the product of reaction with the ~1775 Da peptide fragment. C, The INCA2 reaction gives a peak at ~2536 Da, consistent with hydrolysis of the labile quinoneimine and loss of the –Cl substituents. An overlapping peak at ~2538 Da is resolvable when the data are plotted on an expanded m/z axis (not shown). D, In the INCA6 reaction, at 60 min, the main peak is unreacted peptide and the minor peak at ~2666 Da is the adduct.

Figure 8. Effects of INCA2 on calcineurin-NFAT signalling in cells. A, D5 murine helper T cells were untreated, treated with 1 µM ionomycin alone, or treated with ionomycin in the presence of 4 µM INCA2 or 1 µM CsA and 100 nM FK506. The cytoplasmic or nuclear localization of NFAT1 was visualized by immunocytochemical staining. B, D5 cells were left untreated or pretreated with 4 µM INCA2 or with 1 µM CsA and 100 nM FK506 as indicated, then further incubated for 45 min without stimulation, with 10 nM PMA, or with 10 nM PMA and 300 nM ionomycin. Induction of TNFα and IFNγ mRNAs was analyzed by an RNase protection assay. Lymphotoxin β (LTβ), L32, and GAPDH mRNAs, which are not induced, serve as loading controls. C, D5 cells were treated as in Figure 8B. Induction of MIP-1α and MIP-1β mRNAs was analyzed by RNase protection assay, with RANTES, L32, and GAPDH mRNAs serving as loading controls. The order of lanes is the same as in Figure 8B. D, Dephosphorylation of RII phosphopeptide in lysates from untreated cells (bar 2) or from cells treated with 4 µM INCA2 (bar 3). Total phosphatase activity was assessed by adding the nonspecific inhibitor sodium pyrophosphate (PPi) to the lysate (bar 4). Under the conditions of the assay, essentially all the phosphatase activity measured is due to calcineurin (25, and not shown). No calcineurin activity was detected in lysates from cells incubated with INCA2 for 30 min or 10 min at 37°C (not shown) or, as here, in lysates prepared immediately after addition of INCA2. Data plotted are the means of duplicate samples from a representative experiment.

Figure 9. Structural context of C266 in calcineurin Aα. A, Location of C266 relative to the PxIxIT-binding site, in a model of the calcineurin A (grey)-calcineurin B (black) heterodimer based on coordinates from PDB entry 1AUI. B, Stereo view of the calcineurin-PVIVIT complex (33), indicating the minimum distance from C266 to bound PVIVIT peptide, 15.3 Å. For clarity only calcineurin A residues 260-278, 288-305, and 318-335 are depicted in ribbon representation. C, Stereo view of calcineurin A backbone and sidechain packing in the vicinity of C266. Residues in helix 10 and its immediate flanking segments are shown in white; the bulk of the β11-β12 loop and the initial residues of β strand 12, in lavender; and F299 and P300, in red. The side chains of Y262, V265, C266, and L269 are apposed to the base of the loop that positions residues F299 and P300 to form the PxI-binding subsite, and L275 is packed against F299.
FIGURE 1

INCA1

INCA2

INCA6

INCA12
FIGURE 2

A

```
polarization (mP)
```

```
0°C
RT
bound
free
```

```
time (min)
```

B

```
polarization (mP)
```

```
+DTT
free
```

```
INCA1 (µM)
```

0 1 10 100
FIGURE 4

A

polarization (mP)

INCA12 (μM)

B

polarization (mP)

calcineurin (μM)
FIGURE 5

polarization (mP) vs. INCA2 (µM)

C266A
WT
FIGURE 8

A

CONTROL
IONO
IONO + INCA2
IONO + CsA/FK506

B

LTβ
TNFα
IFNγ
L32
GAPDH

PMA
IONO
INCA2
CsA / FK506

C

RANTES
MIP-1β
MIP-1α
L32
GAPDH

D

phosphate release (cpm)

1 blank
2 control lysate
3 INCA2 lysate
4 control lysate + PPi
FIGURE 9, A and B

A

B

C266

PVIVIT

β11

β12

β14

PxlxIT site

catalytic site

C266

15 Å

15 Å
SUPPLEMENTAL DATA

Supplementary Figure 1. MALDI-TOF analysis of peptide and peptide-INCA1 adduct peaks displayed at higher resolution. A, Peptide at ~2382 Da is resolved into a series of peaks reflecting the natural abundance of the heavy isotopes $^2$H, $^{13}$C, $^{15}$N, $^{18}$O, and $^{34}$S. B, The adduct formed by brief reaction with INCA1 in 0.1% TFA, followed immediately by freezing, is resolved into a similar series of peaks, consistent with a single product of monoisotopic mass ~2732 Da. C, The INCA1 reaction of Figure 7B yielded a more complex pattern indicating an additional product of monoisotopic mass ~2730 Da.
SUPPLEMENTARY FIGURE 1

A

intensity

m/z

B

intensity

m/z

C

intensity

m/z
Inhibition of the calcineurin-NFAT interaction by small organic molecules reflects binding at an allosteric site
Sunghyun Kang, Huiming Li, Anjana Rao and Patrick G. Hogan

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