Aspects of protein phosphatase regulation

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Aspects of protein phosphatase regulation

by

Jennifer Marie Imparl Radosevich

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of the
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Aspects of regulation of two protein phosphatases involved in signal transduction are described in this dissertation. The first phosphatase, calcineurin, is involved in T-cell activation and is also regulated by Ca^{2+}/calmodulin. Because most studies on the interaction between calmodulin and the enzymes which it activates have focused on the role of hydrophobic residues in the interaction, we have instead focused on the role of the positively charged arginine residue in calcineurin-calmodulin interaction. To this end, certain arginine residues in the C-terminal portion of calcineurin were converted to the neutral amino acid citrulline by the action of peptidylarginine deiminase. The resulting loss of phosphatase activity and decreased calmodulin activation of calcineurin was due to an apparent reduced affinity of calmodulin for deiminated calcineurin. We conclude that at least one arginine in calcineurin is important for calmodulin binding and is likely located at the calmodulin binding site of the calcineurin catalytic subunit.

Further localization of the citrulline modifications were attempted by expressing a recombinant calcineurin catalytic
subunit in which the C-terminal 43 residues have been deleted. Deimination of this truncated calcineurin, which lacks four arginine residues, results in the incorporation of 0.5 mol/mol less citrulline than full-length calcineurin and no loss of activity. This may indicate that an arginine not present in the truncated calcineurin is normally deiminated in the full-length calcineurin and results in reduced calmodulin activation and/or binding to full-length calcineurin.

The second phosphatase described in this dissertation is PTP-1, a homolog of a tyrosine phosphatase thought to be involved in the attenuation of the insulin signal. The activity of PTP-1 was found to be inhibited in vitro by compounds derived from cinnamon, compounds which potentiate insulin's effects. One fraction derived from cinnamon is an irreversible inactivator of PTP-1. Another compound which inhibits PTP-1 has a molecular weight of 346. The cinnamon compounds were found to also stimulate insulin receptor kinase catalytic autophosphorylation in vitro. Therefore, the compounds may serve a dual role in insulin signalling. They may act to stimulate insulin receptor signalling by causing activation of its autophosphorylation while inhibiting a PTPase(s) which would attenuate the kinase signal.
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## STUDIES OF THE ROLE(S) OF ARGININE IN THE REGULATORY DOMAINS OF CALCINEURIN

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GENERAL INTRODUCTION

Calcineurin, a calmodulin-activated phosphatase

Calcineurin subunit composition

Calcineurin (CaN) is a major soluble calcium and calmodulin (CaM) binding protein found in brain extracts (1,2). First identified as an inhibitor of calmodulin-stimulated cyclic nucleotide phosphodiesterase because it competed for calmodulin binding (2-4), calcineurin was later shown to have Ca\(^{2+}/\)CaM-regulated phosphatase activity (5,6). A heterodimeric enzyme, calcineurin is composed of a 1:1 complex of a large subunit, CaN A, \(M_r\) 58-61 kDa, and a small subunit, CaN B, \(M_r\) 15-19 kDa (2,7,8). CaN A contains the catalytic site (9) and sole calmodulin binding domain (1), whereas CaN B has 35% sequence identity to calmodulin (10) and binds four calcium ions with micromolar affinity (1). Recently shown to have highly homologous secondary structure to calmodulin (11), CaN B is thought to play a regulatory role in conferring additional calcium sensitivity to the enzyme in vivo (12).

Domain organization of calcineurin

Limited proteolysis of calcineurin by certain proteases has facilitated the domain mapping of CaN A, while CaN B seems resistant to the proteolytic enzymes utilized (13-16). In these experiments, it was shown that the CaN A subunit contains distinct CaN B-binding, CaM-binding, and autoinhibitory domains. The CaN B-binding domain is located on a 44-kDa N-terminal
catalytic core, whereas the CaM-binding and autoinhibitory
domains are found on a 14 kDa C-terminal fragment (16). The
CaM-binding and autoinhibitory domains have also been confirmed
using sequence homology for the CaM-binding domain (17) and
peptide inhibition studies for the autoinhibitory domain (18).
The 44 kDa proteolytic fragment is catalytically active but is
insensitive to activation by Ca\textsuperscript{2+}/CaM (13-16).

**Substrates of CaN**

Calcineurin has been shown to dephosphorylate several
protein substrates (8,19-21), including the $\alpha$-subunit of
phosphorylase kinase and protein phosphatase inhibitor-1 (5),
although the physiological relevance of the dephosphorylation of
any of the substrates surveyed is unknown. In addition to
Ser/Thr phosphatase activity, calcineurin also shows phosphatase
activity toward some phosphotyrosyl proteins and peptides, among
these the epidermal growth factor (EGF) receptor (22), but most
phosphotyrosyl protein are poorer substrates for calcineurin
than their serine or threonine phosphate counterparts (23).
However, in the case of low molecular weight phosphate esters,
phosphotyrosine esters are dephosphorylated readily, while
phosphothreonine and phosphoserine are not significantly
dephosphorylated (24). Perhaps the most useful of the low
molecular weight phosphate esters is p-nitrophenylphosphate
(pNPP), because hydrolysis of pNPP results in the chromogenic
product p-nitrophenol which can be used to monitor calcineurin activity spectrophotometrically.

Another of calcineurin's substrates has received much attention recently because of its apparent role in Alzheimer disease. Aberrant phosphorylation of tau in the Alzheimer condition has been linked to formation of abnormal aggregation of paired helical filaments called neurofibrillary tangles (25,26). Calcineurin has been shown to be able to dephosphorylate this abnormally phosphorylated tau in vitro (27,28), suggesting that regulation of this enzyme could be involved in Alzheimer's. Although the overall levels of the calcineurin protein are not significantly different between Alzheimer and control brain tissue (29), nothing is known about the levels of calcineurin phosphatase activity in the diseased state.

**Regulation of calcineurin by metal ions**

The phosphatase activity of purified calcineurin, in addition to being calmodulin-stimulated, is further stimulated by certain divalent ions, the most efficient of these being Mn\(^{2+}\) and Ni\(^{2+}\) (30-32). There is evidence that Mn\(^{2+}\) and Ni\(^{2+}\) activate the phosphatase activity of calcineurin by different mechanisms. Matsui et al. (33) suggest that Mn\(^{2+}\) and Ni\(^{2+}\) induce different active conformations of calcineurin, since Ni\(^{2+}\)-stimulated calcineurin activity could be inhibited upon addition of a monoclonal antibody specific for CaN B, but Mn\(^{2+}\)-stimulated
calcineurin was not inhibited by the antibody. Perhaps stronger evidence for different conformations of Mn\textsuperscript{2+} and Ni\textsuperscript{2+}-activated calcineurin involves the study of the 44 kDa catalytic core of calcineurin and its regulation by a peptide corresponding to the autoinhibitory region of calcineurin (34). In that study, the autoinhibitory peptide inhibited the Mn\textsuperscript{2+}-stimulated catalytic core by 80%, while the peptide had little effect on the Ni\textsuperscript{2+}-stimulated catalytic core activity. Although no detectable Ni\textsuperscript{2+} or Mn\textsuperscript{2+} can be found in calcineurin immunoprecipitated from bovine brain extracts (35), Ni\textsuperscript{2+} and Mn\textsuperscript{2+} remain to be used routinely in laboratories for calcineurin activity assays.

**Regulation of calcineurin by immunosuppressants**

The involvement of calcineurin in T-cell signalling was confirmed with the discovery that calcineurin is the target of the immunosuppressants FK506 and cyclosporin A (CsA) with their respective receptors, FKBP and cyclophilin (CyP) (36-38). The binding of FK506-FKBP or CsA-CyP complex to calcineurin inhibits its phosphatase activity (37,38) and, in turn, inhibits T-cell activation as assessed by decreased IL-2 production (37,39). It is thought that the inhibition of T-cell activation with immunosuppressant treatment occurs as a result of the inability of the inhibited calcineurin to dephosphorylate NF-AT, a T-cell transcription factor which regulates IL-2 gene synthesis (40). This is because calcineurin has been shown to be able to dephosphorylate NF-AT *in vitro* (41,42), and dephosphorylation of
NF-AT is associated with its translocation from the cytoplasm to the nucleus (41). Consistent with this model, FK506 or CsA blocks NF-AT translocation to the nucleus (41,43), where it can bind DNA and regulate IL-2 production. Conversely, overexpression of calcineurin in Jurkat cells, a human T-cell line, makes them more resistant to FK506 or CsA (44,45) and increases NF-AT dependent transcription (45).

Recently, the sequence and structural aspects of calcineurin required to bind to the immunophilin-drug complex have been studied. The CaN B subunit is absolutely required for binding of drug complex (46,47). However, CaN B is not sufficient for drug complex binding, since minimal sequences in CaN A, sequences in CaN A identified as the CaN B binding site (48), are also required (46,47). The reason for this became apparent when it was found that the "latch region" of CaN B, a region of tertiary structure formed only when CaN B binds CaN A, plays an important role in drug complex binding and CaN A catalytic activation, as determined by site-directed mutagenesis (49). Additional sequences in CaN A also may be important in immunophilin-drug complex binding, since the autoinhibitory domain and FK506-FKBP binding sites on the CaN A subunit appear to overlap (50,51).

**Regulation of calcineurin by calmodulin**

Calmodulin, a ubiquitous calcium-binding protein, binds to and activates several known enzymes (52,53). Like CaN B, it
binds 1 Ca$^{2+}$ ion in each of 4 "EF-hand" binding loops, for a total of 4 mol Ca$^{2+}$/mol (54,55). X-ray structure determination of calmodulin shows that the protein is dumbbell shaped, with two globular domains separated by a central α-helical tether (55). Each globular domain contains 2 EF-hands. While calmodulin is not bound to a target, the central helix is flexible (56). However, when calmodulin is bound to a target peptide of smooth muscle myosin light chain kinase (smMLCK), the central helix is bent, with the two globular domains making various contacts with the peptide (57,58).

Although characterized calmodulin binding domains in calmodulin-activated enzymes show considerable sequence diversity, most are predicted to form basic amphiphilic α-helices (59). The putative calmodulin binding domain of calcineurin is also predicted to form such a helix (17), and there is evidence that calcineurin binds to calmodulin most similarly to smMLCK. For example, crosslinking calmodulin's globular domains together so that the central helix is bent as in the calmodulin-smMLCK peptide complex has no effect on its ability to activate calcineurin (60). Additionally, an especially important sequence of the calmodulin-binding domain of smMLCK, VRAIGR, bears a striking resemblance to a portion of the calmodulin-binding domain of calcineurin, IRAIGK. This extent of sequence homology in calmodulin binding domains is not
observed in comparing the sequences of any other calmodulin-
stimulated enzymes (58).

However, there are clear differences between the mechanism
of binding and activation of calcineurin by calmodulin and that
of some other calmodulin-dependent enzymes. For example, the
autoinhibitory domain of calcineurin is thought to be 50-60
residues C-terminal to the putative CaM-binding domain
(17,18,61). This situation is different from that of smMLCK
(62) and CaM-kinase II (63), where the autoinhibitory region is
adjacent to and slightly overlapping the CaM-binding domain. In
another observation, a mutant calmodulin with 19 amino acid
substitutions could half-maximally activate calcineurin, but
could not activate phosphorylase kinase at all (64).

Many studies on the interaction between calmodulin and the
enzymes which it activates have focused on the role of
hydrophobic residues either in the enzyme (58,65) or in
calmodulin itself (56,57,66,67). X-ray crystal (56) and NMR
solution (57) structures of calmodulin bound to a peptide of
smMLCK show that the binding of calmodulin to its target is
probably dominated by van der Waals forces. However, not much
is known about the importance of electrostatic interactions in
calmodulin-target binding. Although Meador et al. (56) observed
that 80% of the contacts between calmodulin and the smMLCK
peptide were of the van der Waals type, they also noted that all
seven basic residues of the smMLCK peptide make salt bridges with calmodulin.

**Role of arginyl residues in calcineurin**

Chemical modification of arginyl residues by phenylglyoxal has provided limited knowledge of the role(s) arginine plays in calcineurin function (68). A time- and concentration-dependent inactivation of calcineurin activity is seen when calcineurin is reacted with phenylglyoxal. The enzyme is protected from phenylglyoxal inactivation partially by ADP, a competitive inhibitor of calcineurin, and completely by pNPP, a substrate of the phosphatase. This suggests that arginine is important for catalytic activity in or near the catalytic site of the enzyme. Kinetic analysis of the order of the inactivation indicates that only one modified arginine may be responsible for the observed loss of activity. However, inactivation of calcineurin with radioactively labeled phenylglyoxal results in the incorporation of 12 equivalents of phenylglyoxal, making identification of the relevant arginine difficult (68).

**Peptidylarginine deiminase**

Peptidylarginine deiminase has been used to enzymatically convert arginine residues in proteins and peptides to citrulline (69-71). Citrulline is formed from arginine by the loss of a single amino group at the end of the side chain, which results in the loss of the positive charge but does not drastically alter the structure of the side chain. The conversion can cause
significant changes in the function of target protein in vitro (70,71). Because a bulky group, such as phenylglyoxal, is not introduced into the modified protein, effects due to steric hindrance of the modified group on the protein can be discounted. Therefore, peptidylarginine deiminase is a useful probe in studying the role of the positive charge of arginine's guanidino group by conversion of it to a neutral amino acid which cannot be introduced by simple site-directed mutagenesis. The physiological function of peptidylarginine deiminase is still not clear; however, its expression has been found to be regulated by estrogen (72) and the estrous cycle (73,74).

**Insulin signalling and regulation**

**Insulin receptor kinase**

The insulin receptor kinase (IRK) is a heterotetramer, consisting of two extracellular α-subunits disulfide-linked to two β-subunits (αβ)₂. The β-subunits contain a single transmembrane domain, a cytoplasmic tyrosine kinase domain, and a regulatory C-terminal domain (75,76). Insulin binding to the extracellular α-subunits of the insulin receptor kinase presumably causes a conformational change in the tyrosine kinase domain of the β-subunits, resulting in the activation of the insulin receptor's kinase activity. The activation of IRK causes autophosphorylation of the kinase on several tyrosines: Tyr-1158, Tyr-1162, and Tyr-1163 in the kinase region (77-81), Tyr-1328 and Tyr-1334 in the C-terminal tail (77,78), and one or
more of Tyr-965, Tyr-972, and Tyr-984 in the juxtamembrane region (81). These autophosphorylations of IRK on tyrosines are believed to occur via a trans-mechanism, i.e. the kinase domain of one β-subunit phosphorylates tyrosine residues on the adjacent β-subunit (82,83). After autophosphorylation, the insulin receptor kinase is activated toward exogenous substrates (77,79,80).

Recently, the X-ray structure of an inactive form of the kinase domain of the human insulin receptor has been determined (84). The general structure of IRK is similar to that of cAMP-dependent protein kinase (cAPK). The authors observed a smaller N-terminal lobe connected to a larger C-terminal lobe, with a cleft in the middle of the lobes. However, the relative orientation of the lobes in the IRK is quite different from that of cAPK because the N-terminal lobe of IRK is rotated 26° relative to closed-form cAPK and a wider cleft between the two lobes exists. The most striking feature of the crystal structure, though, is the presence of Tyr-1162 in the active site cleft. The tyrosine is not phosphorylated, presumably because the orientation of its binding precludes ATP binding in the active site. The authors postulate, then, that Tyr-1162 binds to the active site of IRK to inhibit its activity while insulin is not bound to the receptor. When insulin is bound to the receptor, then a conformational shift conferred to the β-subunit could displace the inhibitory Tyr-1162, allowing ATP to
bind and tyrosines in the adjacent β-subunit to be phosphorylated.

**Insulin receptor kinase down-regulation and internalization**

As soon as activation of the insulin receptor kinase and propagation of the insulin signal begins, so does down-regulation of the insulin-receptor complex by a process termed "receptor-mediated endocytosis" (85). This means that insulin actually triggers internalization of its own receptor to a network of tubules and vesicles called endosomes. This internalization has been shown to require receptor kinase activation and autophosphorylation on tyrosine, since cells expressing kinase-deficient receptors exhibit impaired insulin internalization (86).

The binding of insulin to its receptor does not occur randomly along the cell surface; instead, the initial binding of insulin occurs on thin digitations called microvilli (87,88). After insulin binding to the receptor, the insulin-receptor complex is redistributed to the nonvillous surface of the cell and is concentrated in clathrin coated pits (87,88). Association of the insulin-receptor complex with clathrin coated pits requires specific sequences in the juxtamembrane domain of the β-subunit (89-91) and sequences in the transmembrane domain itself (92). The insulin-receptor complex then actually enters the cell through invagination of the clathrin coated pit and
fission from the plasma membrane to form an internalized clathrin-coated vesicle (87). Fusion of the complex with the endosome follows. Once the insulin-receptor complex has entered the endosome, the acidic environment of the lumen of the endosome allows dissociation of insulin from the receptor (93,94). The dissociated insulin is targeted to the lysosomes to be degraded (95), while the receptor is recycled back to the cell surface to participate again in insulin signalling (96).

**IRS-1**

A number of proteins in the cell are phosphorylated in response to insulin stimulation (97). One of these, pp185 or insulin-receptor substrate-1 (IRS-1), is a principal substrate of the receptor kinase (98). IRS-1 has not been shown to have any intrinsic enzymatic activity, but seems to constitute a unique signal transduction protein (99). Synthetic peptides based on amino acid sequence of IRS-1 serve as excellent substrates for the receptor kinase *in vitro* (100). The entire amino acid sequence of IRS-1 has been deduced, and it reveals that over 20 potential tyrosine phosphorylation sites exist in the sequence (99). At least 8 of these sites have been shown to be actually phosphorylated by the receptor kinase *in vivo* (101).

**Proteins which interact with IRS-1**

*Phosphatidylinositol 3'-kinase (PI 3'-kinase)*

Phosphatidylinositol 3'-kinase (PI 3'-kinase) was the first protein found to associate with IRS-1 (102-104). PI 3'-kinase
phosphorylates phosphatidylinositol at the D-3 position of the inositol ring (105), but the most important product of PI 3'-kinase may be phosphatidylinositol (3,4,5)-trisphosphate (PIP₃) (106). PIP₃ has been shown to activate protein kinase-C in vitro, indicating a possible second messenger role for PIP₃ (107). PI 3'-kinase itself consists of an 85 kDa regulatory subunit (p85) and an 110 kDa catalytic subunit (p110) (108-111). The regulatory subunit, p85, has been shown to have one Src homology 3 (SH3) domain and two Src homology 2 (SH2) domains (108-110); IRS-1 associates with PI 3'-kinase through the SH2 domains of p85 and activation of the kinase takes place as a result (103,104). It has been reported that IRS-1 becomes serine phosphorylated by PI 3'-kinase during the interaction, but it is not clear what role, if any, this plays in the modulation of insulin signalling (112,113).

Clues into the role(s) of PI 3'-kinase activation in insulin signalling have been obtained through the utilization of two structurally distinct PI 3'-kinase inhibitors, LY294002 and wortmannin. Wortmannin is an antifungal agent which inhibits PI 3'-kinase in the nanomolar range (114,115), while LY294002 is a specific PI 3'-kinase inhibitor based on the bioflavonoid quercetin (116). In Swiss mouse 3T3 fibroblasts overexpressing human insulin receptors, LY294002 partially inhibited glucose uptake, amino acid uptake, and protein synthesis, while it completely inhibited DNA synthesis and p70 S6 kinase activation
The use of wortmannin showed that the activation of PI 3'-kinase during insulin stimulation is a pre-requisite for GLUT 4 translocation and glucose uptake in CHO cells co-expressing human insulin receptors and GLUT 4 glucose transporters (118). These experiments indicate that activation of PI 3'-kinase is important for many aspects of insulin signalling.

GRB-2: linking of p21\textsuperscript{ras} to insulin signalling

GRB-2 is a small molecular weight protein consisting entirely of one SH2 domain and two SH3 domains, but no apparent catalytic domain (119). This "adaptor protein" binds IRS-1 at Tyr-895 after insulin stimulation (119-122). GRB-2 is thought to stimulate p21\textsuperscript{ras} through interaction with one of its SH3 domains with a p21\textsuperscript{ras} GDP/GTP exchange factor called mSOS (121-124). p21\textsuperscript{ras} activation results in the stimulation of the Ser/Thr phosphorylation cascade involving the mitogen-activated protein kinase (MAP kinase) pathway that ultimately leads to S6 kinase activation and cell proliferation (125). S6 kinase also appears to activate glycogen-associated protein phosphatase-1 (PPG-1) which then dephosphorylates glycogen synthase and phosphorylase kinase. This results in the activation of the synthase and inactivation of phosphorylase kinase, with the net effect being glycogen synthesis (126). Therefore, the interaction of tyrosine-phosphorylated IRS-1 with the GRB-2/mSOS complex may contribute to cell proliferation and glycogen synthesis.
SHPTP2 (Syp) SHPTP2, or Syp, is a protein tyrosine phosphatase containing two SH2 domains which binds to IRS-1 in response to insulin (101,127,128). SHPTP2 binding to the phosphorylated consensus motif of YVNI activates its phosphatase activity (129); therefore, binding of SHPTP2 to IRS-1 would presumably activate it in vivo. The downstream signalling of SHPTP2 remains to be elucidated, but SHPTP2 does appear to be a positive regulator of the MAP kinase pathway (130,131).

Nck Nck is a 47 kDa adaptor protein composed of three SH3 domains and one SH2 domain (132), which recently has been shown to bind IRS-1 (133). It is phosphorylated on both serine and threonine residues in response to EGF, PDGF, phorbol esters and cAMP (134-136). However, Nck's role in insulin signalling is unclear.

IRS-1-independent insulin signalling

Shc/GRB-2/mSOS complex formation: insulin sensitive p21ras activation without IRS-1 Shc is another adaptor protein composed of a single SH2 domain, a glycine/proline rich collagen homology domain, and a unique N-terminal domain (137). Shc is tyrosine phosphorylated in response to insulin, which results in the formation of a Shc/GRB-2/mSOS complex (120,138,139). There is growing evidence that activation of p21ras occurs primarily through the Shc/GRB-2/mSOS pathway, rather than the IRS-1/GRB-2/mSOS pathway. First, in CHO cells expressing autophosphorylation-deficient insulin receptors, insulin
stimulated tyrosine phosphorylation on Shc, Shc/GRB-2 formation, and p21ras activation all still occur (140). Second, signalling through the interleukin (IL)-4 receptor, which results in the phosphorylation of IRS-1, but not Shc, fails to activate the MAP kinase pathway (141). Third, perhaps the most compelling evidence that activation of p21ras occurs via Shc/GRB-2/mSOS complex formation comes from mice with a targeted disruption in both copies of their IRS-1 genes ("IRS-1 knockouts") (142). In these mice, the insulin-induced activation of the MAP kinase pathway is observed, although IRS-1 is not present. The MAP kinase pathway is probably activated through Shc, since Shc is normally tyrosine phosphorylated in the mutant mice in response to insulin (142).

"IRS-2" Another group who has described IRS-1 knockout mice has also found evidence for a putative "IRS-2". Araki et al. (143) observed a tyrosine phosphorylated protein ~10 kDa above the position of IRS-1 which immunoprecipitated with PI-3'kinase in mutant mice with no IRS-1. This protein, which does not blot or immunoprecipitate with antibodies against IRS-1, has been named "IRS-2". IRS-2 may act as an alternate substrate of the insulin receptor and may substitute for IRS-1 in binding to and activating PI 3'-kinase, since insulin-stimulated PI 3'-kinase activation was observed in mutant mice and the IRS-2 protein immunoprecipitated down with PI-3'kinase. Future
research in insulin signalling most certainly will include characterization of the IRS-2.

**Role of PTPases in insulin signal transduction**

**General evidence for role(s) of PTPases in insulin signalling**

There is substantial evidence supporting a role(s) for protein tyrosine phosphatases (PTPases) in insulin signalling; however, specific roles for specific phosphatases have not yet been confirmed. Microinjection of PTP-1B into Xenopus oocytes blocks insulin-stimulated S6 peptide phosphorylation and retards insulin-induced oocyte maturation (144,145), but it is not certain at what point(s) in the insulin signalling pathway the phosphatase is having the observed effect in this system. IRS-1 is phosphorylated by the activated receptor within seconds of insulin stimulation, but this phosphorylation is transient (146,147). The subsequent dephosphorylation of IRS-1 occurs despite continued insulin stimulation (147). Nothing is known about the specific PTPase(s) involved in this process.

More is known about the roles that PTPases play in dephosphorylating the insulin receptor itself. In cultured cells and permeabilized adipocytes, dissociation of insulin from its receptor is followed by a rapid dephosphorylation of the receptor β-subunit and deactivation of the kinase (148-150). Additionally, vanadate and other related compounds which are known to inhibit PTPases enhance the phosphorylation state of
the insulin receptor and certain cellular substrate proteins, resulting in increased insulin signalling (151-154). These data suggest that PTPases which dephosphorylate the autophosphorylated insulin receptor may act to attenuate the insulin signal in vivo.

**Possible PTPase candidates for the dephosphorylation of insulin receptor kinase in vivo**

Known PTPases seem to fall into two broad categories, the receptor-type and non-receptor type. The receptor-type PTPases are composed of a glycosylated extracellular domain, a single transmembrane region, and one or two conserved catalytic domains (155,156). Examples of this type of PTPase are LAR (157,158), CD45 or LCA (158,159), and LRP (160). The non-receptor type has no extracellular or transmembrane domains, but typically a single catalytic region with additional domains with other functional properties (156). Examples of the non-receptor type PTPases are PTP-1B (161,162) and the T-cell PTPase (163).

Clues into which PTPases may act to attenuate the insulin signal come from studies of the phosphorylation state of the kinase region of the insulin receptor kinase. Autophosphorylation of all three tyrosines in the kinase domain (Tyr-1146, 1150, and 1151) is required for full activation of phosphotransferase activity in an in vitro insulin receptor kinase assay (164,165). However, the receptor is frequently isolated from insulin-stimulated cells in the mono- or bis-
phosphorylated form, suggesting that receptor autophosphorylation is closely regulated by phosphatases in vivo (165,166). Additionally, the initial dephosphorylation of the tris-phosphorylated form of the receptor kinase on either Tyr-1150 or Tyr-1151 correlates with the deactivation of kinase activity (167). Therefore, studies were undertaken to determine which PTPases would preferentially dephosphorylate these tyrosines in the autophosphorylation domain of the insulin receptor.

In vitro, PTPase will dephosphorylate a number of protein and peptide substrates, although with different kinetic parameters (155,159,160,163,168). Purified PTP-1B and CD45 are active against the autophosphorylated insulin receptor in vitro (158,159,162,169), while the catalytic domains of PTP-1B, LAR, and LRP expressed in recombinant bacterial systems are also active against the receptor in vitro (170,171). However, differences in dephosphorylation kinetics and regiospecificity exist among these phosphatases. For example, PTP-1B dephosphorylates Tyr-1150 on the tris-phosphorylated insulin receptor 4 times faster than the mono- or bis-phosphorylated receptor (169). In another study which utilized an insulin receptor related phosphopeptide corresponding to the autophosphorylated kinase domain, the authors observed preferential dephosphorylation of the three phosphotyrosines (172). In that study, PTP-1B preferentially dephosphorylated
Tyr-1150 or Tyr-1151, LAR and CD45 preferred Tyr-1146, while the T-cell PTPases exhibited no specificity for a particular phosphotyrosine.

Although Ramachandran et al. showed that LAR preferentially dephosphorylated Tyr-1146 on a tris-phosphorylated kinase domain peptide, other studies suggest that LAR actually preferentially dephosphorylates Tyr-1150 in intact insulin receptor (171) and may be important in attenuating insulin receptor kinase activity (173). Hashimoto et al. (171) observed the deactivation of the insulin receptor by LAR, LRP, and PTP-1B. LAR deactivated the receptor kinase two to three times more rapidly than LRP or PTP-1B, and the tryptic map of the receptor β-subunit after dephosphorylation by LAR showed preferential dephosphorylation of Tyr-1150. Additional evidence that LAR may be involved in the modulation of insulin signalling in intact cells comes from a rat hepatoma cell line that had been made to express LAR antisense RNA (173). In these cells, LAR protein levels were suppressed by 63%, which increased insulin-dependent autophosphorylation of the insulin receptor by 150%, and, in turn, insulin-dependent PI 3'-kinase by 350%. Since LAR is prominently expressed in rat liver (174), it is a strong candidate for being a PTPase that may be involved in insulin signalling.

Another strong candidate PTPase which may be involved in dephosphorylation of the insulin receptor in vivo is PTP-1B. As
mentioned before, PTP-1B activity against a phosphopeptide encompassing the autophosphorylation domain of the insulin receptor β-subunit results in the preferential dephosphorylation of Tyr-1150 or Tyr-1151 (172), sites critical for deactivation of the receptor kinase (167). When PTP-1B and insulin receptors were co-expressed to high levels, insulin receptor autophosphorylation was inhibited (175). Even PTP-1B mRNA levels are responsive to insulin. Treatment of rat hematoma cells with 100 nM insulin increases the abundance of the two mRNA's encoding PTP-1B by 1.6- and 3.1-fold, with no significant effect on the abundance of mRNA encoding LAR or LRP (174). Therefore, PTP-1B may provide feedback regulation of insulin signalling. Future research concerning the roles of PTPases in insulin signalling in vivo most certainly will involve further characterization of PTP-1B and LAR in this pathway.

Diabetes and PTPase activity Severe obesity or non-insulin dependent diabetes mellitus (NIDDM) is characterized by the diminished ability of insulin-dependent tissues to respond to insulin (176). This insulin-resistance could be the result of a number of defects in the insulin signalling pathway (177-181). However, much focus has been put on evidence that insulin receptor kinase autophosphorylation and subsequent IRS-1 phosphorylation are markedly decreased in insulin-responsive tissues of subjects with severe obesity or NIDDM (182-184). Recently, Nadiv et al. (185) have shown that this decreased
degree of phosphorylation of the insulin receptor may be due to the action of protein tyrosine phosphatases. The authors observed elevated cytosolic PTPase activity directed against the β-subunit of the insulin receptor in aged, insulin-resistant rats. Begum et al. (186) also reported increased PTPase activity toward intact insulin receptors in diabetic rat adipocytes, although they found the elevated PTPase activity in the particulate fraction. Regardless of the subcellular localization of the PTPases involved, enhancement of PTPase activity toward the insulin receptor kinase in insulin-dependent tissues would apparently result in impaired insulin receptor signalling in those tissues.

The development of oral anti-diabetic agents which enhance insulin sensitivity in insulin-dependent tissues has received much attention (187-189). One anti-diabetic agent, pioglitazone, has been shown to increase insulin sensitivity through its ability to reactivate insulin receptor kinase autophosphorylation (190,191). Pioglitazone does not act directly on the receptor, since incubation of insulin receptors and pioglitazone in vitro does not result in increased receptor kinase autophosphorylation (190). There is now strong evidence that this indirect effect of pioglitazone on receptor autophosphorylation is due, at least in part, to normalization of elevated PTPase activities in insulin-unresponsive cells (192). In this study, Rat 1 fibroblasts overexpressing the
human insulin receptor were made impaired in insulin signalling with treatment with high glucose; decreased phosphorylation of the insulin receptor and IRS-1, along with elevated PTPase activity, were associated with the impaired signalling. Pioglitazone treatment reversed all these defects in insulin signalling, apparently through decreasing the activity of the elevated cytosolic PTPase activities. Preliminary evidence suggests that the cytosolic PTPase responsible for those effects is PTP-1B (192). Therefore, some anti-diabetic agents may act to decrease elevated PTPase activities in insulin-dependent tissues, restoring insulin sensitivity to those tissues by allowing autophosphorylation of the insulin receptor and resultant insulin signal transduction.

**Nutrition, glucose tolerance, and insulin signalling**

In 1959, chromium was shown to be required for maintaining normal glucose tolerance in rats (193). This lead to the notion that a slight chromium deficiency in humans could mean an increased risk of developing diabetes (194). Evidence to support this hypothesis came from two double-blind, crossover studies in humans, where increased glucose tolerance was observed upon chromium supplementation of subjects with mildly impaired glucose tolerance (195,196).

There is mounting evidence that the chromium responsible for improved glucose tolerance is not that of a simple inorganic chromium, such as chromium chloride, but that the chromium must
be complexed with certain ligands to exert its biological activity (194,197,198). There are two known biological chromium complexes which have received much attention. The first, termed glucose tolerance factor (GTF), was originally extracted from Brewer's yeast and was found to contain chromium, nicotinic acid, glycine, glutamic acid, and cysteine (199). A synthetic mixture of the components of GTF is nearly identical to Brewer's yeast GTF in its physical characteristics and biological activity (199). Furthermore, the synthetic mixture mimicks the effects of extracted GTF by lowering plasma glucose concentrations in genetically diabetic mice (198). The second biologically active chromium complex is known as low-molecular weight chromium binding ligand (LMWCr), which has been isolated from bovine colostrum (200). Having a molecular weight of ~1500, LMWCr contains chromium, aspartic acid, glutamic acid, and cysteine, but no nicotinic acid. LMWCr has been shown to exhibit biological activity, as detected by glucose oxidation in isolated fat cells, similarly to that of GTF (200).

Some extracts of certain spices also exhibit insulin potentiating activity in the rat epididymal fat cell assay (201,202). One of the spices which best potentiates insulin's effects in these cells is cinnamon (201). It is not known how cinnamon exerts its effect. It apparently is not due to the action of biological chromium in the spice, since chromium concentration in spices is not correlated with their insulin
potentiating activity (201). A number of phenols, aldehydes, and acids have been identified in extracts of cinnamon, including eugenol, cinnamaldehyde, and cinnamic acid (203). It remains to be seen if any of these identified compounds are responsible for cinnamon's biological activity, or if a previously unidentified compound is responsible.

Dissertation organization

This dissertation consists of three papers which follow the general introduction. The first paper was published in the Archives of Biochemistry and Biophysics and describes the role of arginine residues in the interaction between calcineurin and calmodulin. The second paper is to be submitted for publication and is a continuation of studies in the first paper, describing the role(s) of arginine in the regulatory domains in calcineurin. The third paper is to be submitted for publication and deals with the regulation of another phosphatase, protein tyrosine phosphatase-1 (PTP-1), by compounds derived from cinnamon. The general summary and discussion follows the third paper, while the references cited in the general introduction and general summary follow the general summary.
STUDIES OF CALCINEURIN-CALMODULIN INTERACTION:
PROBING THE ROLE OF ARGinine RESIDUES USING
PEPTIDYLARGININE DEIMINASE

A paper published in Archives of Biochemistry and

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Abbreviations

CaN, calcineurin;
CaM, calmodulin;
EGTA, Ethylene glycol bis(β-amino ethyl ether)N,N'-tetraacetate;
pNPP, para-Nitrophenyl Phosphate;
pNP, para-Nitrophenol;
MOPS, 3-(N-morpholino)propanesulfonic acid;
PVDF, polyvinylidene difluoride;
HRP, horseradish peroxidase;
SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis;
smMLCK, smooth muscle myosin light chain kinase

Abstract

We have used an enzyme, peptidylarginine deiminase, to convert certain arginyl groups in calcineurin to citrulline. Amino acid analysis shows that only 3 out of 34 arginines in calcineurin were deiminated; citrulline seems to be localized only in the calcineurin A (CaN A) subunit. Upon incubation with deiminase, the Mn^{2+}/calmodulin stimulated phosphatase activity decreases to 20-40% of the original activity within one hour. However, the reduction in enzyme activity is fully protected by addition of calmodulin to the deimination reaction, and only 1.5 mol citrulline/mol calcineurin is found in this case. Removal of the calmodulin binding domain of the deiminated CaN A by limited proteolysis results in the reactivation of the
phosphatase to the same level as digested native calcineurin and also results in the loss of all citrulline residues. The calmodulin activation curve of the deiminated enzyme is significantly shifted; the calculated apparent $K_{\text{act}}$ using native calmodulin is 15-fold higher than that of native calcineurin while the apparent $K_{\text{act}}$ using a fluorescent derivative of calmodulin, dansyl-calmodulin, is 10-fold higher. However, the $V_m$ of deiminated calcineurin is similar to that of native, if highly elevated levels of calmodulin are used to activate the modified calcineurin. To determine directly if the binding of calmodulin to calcineurin is affected upon deimination, fluorescence titrations using dansyl-calmodulin were performed. The $K_d$ of deiminated calcineurin determined from these titrations is 10-fold higher than that of unmodified calcineurin, indicating that calmodulin binding is indeed affected. These data indicate that at least one arginine is important for calmodulin binding and is likely located at the calmodulin binding site of the CaN A subunit.

**Introduction**

Calcineurin (CaN) is a major soluble calcium and calmodulin binding protein found in brain extracts (1,2). First identified as an inhibitor of calmodulin-stimulated cyclic nucleotide phosphodiesterase because it competed for calmodulin binding (2-4), calcineurin was later shown to have Ca$^{2+}$/CaM regulated phosphatase activity (5,6). This activity has been shown to be inhibited by a complex of the immunosuppressant
drugs cyclosporin A or FK506 and their respective intracellular receptors (7-9). A heterodimeric enzyme, calcineurin is comprised of a 1:1 complex of a large subunit, CaN A, Mr 58-61 kDa, and a small subunit, CaN B, Mr 15-19 kDa (2,10,11). CaN A contains the catalytic site (12) and sole calmodulin binding domain (1) whereas CaN B has 35% sequence identity to calmodulin (13) and binds 4 calcium ions with micromolar affinity (1). Recently shown to have highly homologous secondary structure to calmodulin (14), CaN B is thought to play a regulatory role in conferring additional calcium sensitivity to the enzyme in vivo (15).

Limited proteolysis of calcineurin by certain proteases has facilitated the domain mapping of CaN A, while CaN B seems resistant to the proteolytic enzymes utilized (16-19). In these experiments, it was shown that the CaN A subunit contains distinct CaN B-binding, CaM-binding, and autoinhibitory domains. The CaN B-binding domain is located on a 44 kDa N-terminal catalytic core, whereas the CaM-binding and autoinhibitory domains are found on a 14 kDa C-terminal fragment (19). The CaM-binding and autoinhibitory domains have also been confirmed using sequence homology for the CaM-binding domain (20) and peptide inhibition studies for the autoinhibitory domain (21). The 44 kDa proteolytic fragment is catalytically active but is insensitive to activation by Ca\(^{2+}\)/CaM (16-19).
Chemical modification of arginyl residues by phenylglyoxal has provided limited knowledge of the role(s) arginine plays in calcineurin function (22). A time and concentration dependent inactivation of calcineurin activity is seen when calcineurin is reacted with phenylglyoxal. The enzyme is protected from phenylglyoxal inactivation partially by ADP, a competitive inhibitor of calcineurin, and completely by pNPP, a substrate of the phosphatase. This suggests that arginine(s) are important for catalytic activity in or near the catalytic site of the enzyme. Kinetic analysis of the order of the inactivation reaction indicates that only one modified arginine may be responsible for the observed loss of activity. However, inactivation of calcineurin with radioactively labeled phenylglyoxal results in incorporation of 12 equivalents of phenylglyoxal, making identification of the relevant arginine difficult (22).

Peptidylarginine deiminase has been used to enzymatically convert arginine residues in proteins and peptides to citrulline (23,24). Citrulline is formed from arginine by the loss of a single amino group at the end of the side chain, which results in the loss of the positive charge but does not drastically alter the structure of the side chain. Because a bulky group, such as phenylglyoxal, is not introduced into the modified protein, effects due to steric hindrance of the modified group on the protein can be discounted. Therefore, peptidylarginine deiminase is a useful probe to study the role of the positive
charge of arginine's guanidino group by conversion of it to a neutral amino acid which cannot be introduced into proteins by simple site-directed mutagenesis. The present paper deals with the study of a modified form of bovine brain calcineurin in which certain arginyl groups have been converted to citrulline by the deiminase, resulting in a marked reduction in the enzyme's phosphatase activity and binding affinity for calmodulin. We find evidence suggesting that modification of an arginine residue(s) localized in the calmodulin binding site is responsible for these observations; therefore, this approach may prove useful to further characterize calcineurin-calmodulin interaction.

**Experimental Procedures**

**Materials**

All buffers, as well as pNPP, EGTA, and phenyl-Sepharose, were purchased from Sigma. All other chemicals were from Fisher. PVDF membrane and HRP-conjugated goat anti-rabbit IgG were from BioRad. Antibody recognizing modified citrulline residues in proteins has been described previously (25). ECL chemiluminescent reagents were purchased from Amersham (Arlington Heights, IL). 3,3'-diaminobenzidine tetrahydrochloride (DAB) was from Gibco BRL.

**Enzymes**

Calcineurin was purified from bovine brain by minor modification of the method of Sharma et al. (26). Calmodulin was also from bovine brain using the method of Sharma and Wang (27) modified to include phenyl-Sepharose chromatography after DE-52 chromatography, according to
Gopolakushna and Anderson (28). Peptidylarginine deiminase was purified from frozen rabbit skeletal muscle, according to Takahara et al. (29). Clostripain was purchased from Sigma. Protein concentration was determined by the method of Bradford (30).

**Calcineurin assays** Using pNPP as substrate, calcineurin was assayed continuously by measuring the pNP at 410 nm in a Beckman DU-7 spectrophotometer. Temperature was maintained at 30°C using a Peltier temperature control element. Typical assays were done in 25 mM MOPS, pH 7.0, 1 mM MnCl₂ or 0.5 mM NiCl₂, 20 mM pNPP, 10 μg/ml calmodulin, and 10 μg/ml calcineurin. The concentrations of metal ion used in each case were determined to be saturating (data not shown).

**Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis, Electroblotting, and Western Analysis for Citrulline**

Calcineurin samples were separated by SDS-PAGE (31) on a 12.5% polyacrylamide gel and electroblotted onto PVDF using 10 mM CAPS, pH 11.0, in 10% methanol or onto nitrocellulose using 25 mM Tris, pH 8.3, and 192 mM glycine in 20% methanol (constant 300 mA for 1 hour). The membrane was either stained for protein with 0.025% (w/v) Coomassie Brilliant Blue R250 in 50% methanol (v/v) or processed for western analysis using an anti-modified-citrulline antibody (25). Before western analysis, the blotted membrane was incubated at 37°C in modification medium (1 vol ddH₂O, 1 vol 2% diacetyl monoxime/1% antipyrine/1 N acetic acid,
and 2 vol 85%(w/w)H₃PO₄/98%(w/w)H₂SO₄/H₂O (20/25/55, v/v)) to modify citrulline residues. After rinsing with ddH₂O, the membrane was soaked in blocking medium (5% skim milk in 10 mM Tris-HCl, pH 7.5, 0.14 M NaCl, 0.1% Tween 20 (TBST)) for 1 hour, then the antibody (0.125 µg/ml) was added and the blot incubated for 2 more hours. The membrane was washed with 3 X 10 min with TBST, soaked in blocking medium for 30 min, and soaked in HRP-goat anti-rabbit IgG (1:4000) for 1 hour. After washing with TBST as before, detection was performed either by addition of DAB/H₂O₂ to the blot or according to the ECL protocol of Amersham.

**Amino Acid Analysis** Protein bands (CaN A and CaN B) separated by SDS-PAGE and electroblotted onto PVDF were excised from the membrane and subjected to amino acid analysis. The analysis was performed on an Applied Biosystems 420A Amino Acid Analyzer by the Iowa State University Protein Facility. Quantitation of citrulline was determined from the citrulline plus ornithine peaks in the HPLC trace, because degradation of citrulline into ornithine occurs during the acid hydrolysis step (23).

**Fluorescence Measurements and Kₐ Calculations** Dansyl-CaM, with an incorporation of 1 mol dye/mol CaM, was prepared as described by Kincaid et al. (32). The buffer used for the fluorescence experiments was 25 mM MOPS, pH 7.0, containing 1 mM MnCl₂. Fluorescence emission spectra and titration of dansyl-
CaM and calcineurin were done at an excitation wavelength of 340 nm using a SPEX fluorolog 2 spectrophotometer. For titration experiments, total fluorescence intensity was measured at an emission wavelength of 500 nm. Fluorescence data were obtained 60 seconds after addition of titrant, of which concentrated solutions were added to keep dilution below 5%. Intensity data were corrected for dilution and background fluorescence of protein and solvent.

Kd of CaN-CaM was calculated from total fluorescence data using the method of Malencik and Anderson (33). By measuring the fluorescence intensity of fully bound (F∞) and unbound (Fo) ligand (dansyl-CaM), and then measuring fluorescence intensity values during titration (F), a fractional saturation (Φ) of dansyl-CaM can be calculated for each titration point:

\[
Φ = \frac{(F/Fo-1)}{(F∞/Fo-1)}
\]

Knowing Φ, [CaN]free can be calculated. A hyperbolic plot of F/Fo vs. [CaN]free then was done using Enzfitter by Elsevier-Biosoft, and this yielded the calculated Kd.

Results

Effect of deimination of calcineurin on phosphatase activity When calcineurin was incubated with peptidylarginine deiminase, the Mn²⁺/CaM-stimulated enzymatic activity of calcineurin was reduced to 20-40% of the original
activity within one hour (Fig. 1). Further incubation of calcineurin with deiminase resulted in no additional loss of activity (data not shown). Addition of calmodulin to the reaction mixture before addition of deiminase, however, fully prevented the decrease in calcineurin activity (Fig. 2A). This observed effect could be due to calmodulin protecting calcineurin from modification(s) introduced directly into calcineurin by the deiminase, but it also could be due to interference of calcineurin deimination by preferential deimination of calmodulin. To distinguish between these two possibilities, calmodulin preincubated with the deiminase was used in a standard calcineurin assay to determine its ability to activate calcineurin. As seen in Figure 2B, preincubation with deiminase had no effect on calmodulin's ability to activate calcineurin. Additionally, we found no evidence that calmodulin was a substrate for the deiminase, as determined by western analysis (described later in Fig. 3A, lane 4). These data indicate that calmodulin probably protected calcineurin by forming a complex with it and preventing a certain modification(s) by the deiminase.

Localization of citrulline residues in calcineurin

The above calmodulin protection result suggested that one or more arginines important for calmodulin binding or activation were modified by the deiminase. The localization of converted arginines in modified calcineurin has been facilitated by
Figure 1. Time course of incubation of calcineurin with deiminase. Control CaN (■) and CaN + deiminase (●) were incubated at 37°C in a buffer containing 50 mM Hepes, pH 7.2, 5 mM CaCl₂, 5 mM DTT with or without 80 μg/ml deiminase, then aliquots were removed and assayed immediately at the designated time points. Calcineurin concentration for the deimination reaction was 1.1 mg/ml.
Figure 2. Calmodulin protection of the reduction in phosphatase activity of calcineurin during incubation with peptidylarginine deiminase. (A) Reaction was performed as in Figure 1, except that calmodulin (42 μM, 3-fold molar excess over calcineurin) was included in the deimination reaction. Control CaN (■), CaN + deiminase (○), control CaN + CaM (▲), and CaN + deiminase + CaM (★). Enzyme velocities were normalized to 100% based on the velocity of the first assay point at time 0; actual velocities in μmol/min/mg were: control CaN, 2.4, CaN + deiminase, 2.8, CaN + CaM, 2.1, and CaN + deiminase + CaM, 2.3. (B) 42 μM calmodulin was incubated at 37°C in the absence (♦) or presence (○) of 80 μg/ml deiminase. Then, the calmodulin was used in a standard CaN assay (see Experimental Procedures) at the times indicated to determine its ability to activate calcineurin.
A.
B.

(figure 2 continued)
Figure 3. Localization of citrulline residues in calcineurin.

(A) Reactions were performed as in Figure 2, then 5 μg CaN per lane was loaded onto a 12.5% SDS-PAGE gel. After electrophoresis, the separated subunits were electroblotted onto PVDF and analyzed for the presence of citrulline using an anti-citrulline antibody (see Experimental Procedures). Lanes are: (1) Control CaN (2) Control CaN + CaM (3) CaN + deiminase (4) CaN + deiminase + CaM. Lanes 5 and 6 are Control CaN (5) and CaN + deiminase (6) to which 2 U of clostripain performed as in (A), then 5 μg CaN per lane was loaded onto a 12.5% SDS-PAGE gel. (B) After electrophoresis, the gel was stained with Coomassie blue. Lanes are: (1) molecular weight markers, 97, 66, 45, 31, 22, and 14 kDa (2) undigested CaN (3) digested CaN. Proteolysis results in the formation of a 45 kDa N-terminal CaN A fragment with no calmodulin binding domain (19).
A.

1 2 3 4 5 6

61 kDa
44 kDa
19 kDa
(figure 3 continued)
electroblotting separated samples on an SDS-PAGE gel onto PVDF followed by western analysis using an anti-modified-citrulline antibody (25). The western analysis revealed that only the CaN A subunit contained citrulline (Fig. 3A), even after detection with the extremely sensitive chemilluminescent ECL (Amersham) technique and overexposure of the film (data not shown). Peptides of molecular weight lower than 61 kDa, but higher than that of calmodulin, were found to contain citrulline (Fig. 3A, lanes 3 and 4); these are presumably degradation products generated during the preparation of calcineurin. Since the deiminase was found to deiminate itself (not shown), an accurate determination of the incorporation of citrulline into calcineurin was carried out by amino acid analysis on the CaN A and CaN B bands separated from the deiminase by SDS-PAGE and electroblotted onto PVDF. Two independent determinations yielded 2.6 and 3.3 mol citrulline/ mol CaN A subunit. Therefore, approximately 3 arginine residues are modified by the deiminase out of a possible 28 arginines in the catalytic subunit.

Hubbard and Klee have shown that limited digestion of calcineurin by clostripain results in the formation of a 44 kDa N-terminal catalytic domain of CaN A which has CaM-independent phosphatase activity, while the CaN B subunit is not cleaved (19). Upon proteolysis, they observed Ca^{2+}/CaM-independent phosphatase activity 3-fold higher than that of native CaM-dependent activity, because the 14 kDa C-terminal region of CaN
A cleaved from the N-terminal 44 kDa fragment contains both the calmodulin binding and autoinhibitory domains. To determine if any or all of the modified residues in deiminated CaN A were in the C-terminal region, we performed a limited proteolysis of deiminated calcineurin by clostripain (34,35). This yielded a 44 kDa N-terminal fragment (Fig. 3B) in which no citrulline was detected either by western (Fig. 3A, lane 6) or amino acid analysis. A citrulline positive 14 kDa fragment was not seen on the gel, since the fragment is further proteolyzed once cleaved from the intact protein (19). When the CaM-independent activity of the 44 kDa catalytic fragment was measured, the deiminated calcineurin digest showed comparable activity to that of the unmodified calcineurin digest. (Fig. 4). Therefore, the loss of citrulline and the calmodulin binding domain from the 44 kDa fragment upon limited proteolysis is accompanied by reactivation of deiminated phosphatase activity. As observed by Hubbard and Klee, 3-fold activation of native CaM-dependent activity is seen (Fig. 4) because the proteolysis results in the cleavage of the autoinhibitory domain as well as the CaM binding domain (19,21).

An interesting observation is that, although addition of calmodulin to the deimination reaction prevented loss of the CaM-dependent phosphatase activity of calcineurin, the western analysis showed deimination of CaN A without detectable deimination of calmodulin (Fig. 3, lane 4). Amino acid analysis of calcineurin A obtained after preincubation with calmodulin and the deiminase gave 1.4 and 1.7 mol citrulline/mol in two
Figure 4. Reactivation of deiminated calcineurin upon limited proteolysis with clostripain. 5.2 U of clostripain was added to 21 μg unmodified or deiminated calcineurin and allowed to react 10 seconds. Digested control and deiminated calcineurin was assayed in the presence or absence of calmodulin. (Closed bars, unmodified CaN, open bars, deiminated calcineurin).
independent determinations. This indicates that one or two of
the modifications taking place during the deimination reaction
are irrelevant to the loss of activity seen when calmodulin is
not included in the reaction. Therefore, deimination of only
one or possibly two of the three modified arginine residues in
CaN A may be necessary for the loss of calcineurin activity upon
its deimination in the absence of calmodulin. An alternative
explanation is that more than two arginines are partly modified,
and all of these changes contribute to the inactivation.

**Kinetic and activation constants** Using a range of 8-80
mM of the substrate pNPP and a constant concentration (10 µg/ml)
of calmodulin, native calcineurin exhibited a \( K_m \) of 30.2 mM and
a \( V_m \) of 2.56 µmol P_i/min/mg (Table I). After modification with
deiminase, the \( K_m \) for pNPP was relatively unchanged (38.9 mM),
while the \( V_m \) was half the value (0.96 µmol P_i/min/mg). However,
when the phosphatase activity of unmodified and modified
calcineurin was measured using increasing concentrations of
calmodulin at constant pNPP, the deiminated calcineurin showed a
similar maximum velocity as that of native, if high enough
concentrations of calmodulin were used to activate the
deiminated calcineurin (closed circles of Fig. 5). Indeed, the
\( V_m \) of deiminated calcineurin was measured to be similar to that
of native, if saturating calmodulin was used to activate each
form of calcineurin (Table I). Still, the calculated apparent
\( K_{act} \) of deiminated calcineurin by calmodulin was 15-fold higher
than the apparent \( K_{act} \) of native (Table II), indicating that
Table I. Kinetic parameters of calcineurin after deimination. Notes. Control or deiminated calcineurin was assayed (A) with a constant 10 µg/ml calmodulin and a range of 8–80 mM pNPP or (B) saturating calmodulin conditions for CaN (20 µg/ml CaM) and deiminated CaN (500 µg/ml CaM) with a range of 8–200 mM pNPP. Saturating calmodulin conditions for control and deiminated calcineurin were determined from Figure 5; these concentrations of calmodulin activated both of these forms of the enzyme with a similar velocity of reaction using pNPP. $V_m$ differs between (A) and (B) for control CaN because these determinations were made using two different preparations of CaN. Kinetic parameters and errors were determined with Enzfitter computer software by Elsevier-Biosoft.

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<th>Km (mM)</th>
<th>$V_m$ (nmol Pi/min/mg)</th>
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<tr>
<td><strong>A. 10 µg/ml CaM</strong></td>
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<tr>
<td>CaN</td>
<td>30.2 ± 2.0</td>
<td>2.56 ± 0.07</td>
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<tr>
<td>CaN + deiminase</td>
<td>38.9 ± 10.2</td>
<td>0.96 ± 0.12</td>
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<td><strong>B. saturating CaM</strong></td>
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<tr>
<td>CaN</td>
<td>38.4 ± 4.1</td>
<td>1.36 ± 0.07</td>
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<tr>
<td>CaN + deiminase</td>
<td>82.0 ± 12.2</td>
<td>1.16 ± 0.08</td>
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Figure 5. Activation curves of native and deiminated calcineurin with native and dansyl-calmodulin. Calcineurin preincubated at 37°C for 1 hour without or with deiminase was checked for reduction in activity, then placed on ice. Aliquots were then taken and assayed for activity using increasing concentrations of calmodulin or dansyl-calmodulin. (CaN + CaM (■); CaN + dansyl-CaM (▲); deiminated CaN + CaM (●); deiminated CaN + dansyl-CaM (★)).
Table II. Activation and dissociation constants of control and deiminated calcineurin with calmodulin. Notes. Activation ($K_{act}$) and dissociation ($K_d$) constants were determined from the data in Figures 5 and 6 using the Enzfitter computer software by Elsevier-Biosoft. The apparent $K_{act}$ of CaN by CaM was calculated without correction for CaN concentration to facilitate comparison of the value obtained with those found in the literature, which were calculated similarly. The apparent $K_{act}$ obtained for control CaN obtained in this study (37 nM) agrees well with the range of values found in the literature (16-100 nM, references 11, 53-56). Although methodology differs greatly for $K_d$ determinations, the $K_d$ obtained in this study (5.5 nM) also agrees with values found in the literature (0.2-8.0 nM, references 2, 57, 58).

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<th>$K_{act}$ (nM)</th>
<th>$K_d$ (nM)</th>
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<tr>
<td></td>
<td>CaM</td>
<td>dansyl-CaM</td>
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<tr>
<td>CaN</td>
<td>37 ± 7</td>
<td>34 ± 10</td>
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<tr>
<td>CaN + deiminase</td>
<td>657 ± 240</td>
<td>325 ± 120</td>
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extremely high concentrations of calmodulin were needed to activate deiminated calcineurin.

**Binding constants** Since calmodulin's ability to activate calcineurin decreased upon deimination of calcineurin, it was necessary to know whether the problem lay in calmodulin's activation of calcineurin or in the binding of calmodulin to calcineurin. To distinguish between these two possibilities, a fluorescent derivative of calmodulin, dimethylaminonaphthalene (dansyl)-calmodulin, was employed. Dansyl-calmodulin undergoes a Ca^{2+}-dependent increase in fluorescence intensity which is enhanced when it binds calcineurin (36). By measuring the increase in fluorescence intensity during the dansyl-CaM-calcineurin titration, the dissociation constant, K_d, can be found.

To insure that dansyl-calmodulin could bind to calcineurin similarly to native calmodulin, its ability to activate calcineurin was measured. Although the maximal velocity of calcineurin activated by dansyl-calmodulin was only 60-70% of that of calcineurin activated by native calmodulin (Fig. 5), a comparison of the apparent K_{act} and K_d of native vs. deiminated calcineurin was desired. Dansyl-calmodulin activated calcineurin with a similar K_{act} to the non-fluorescent derivative (Fig. 5 and Table II), allowing such a comparison. A 10-fold difference in the apparent K_{act} was observed between the native and deiminated forms of calcineurin when dansyl-calmodulin was used as an activator (Table II). Similarly, a
10-fold difference in $K_d$ for dansyl-calmodulin was found between native and deiminated calcineurin (Fig. 6 and Table II). This indicates that the deimination of calcineurin indeed affects binding of calmodulin to calcineurin and that the 10-fold difference in the apparent $K_{act}$ between native and deiminated forms of calcineurin is likely due to a comparable decrease in calmodulin binding to calcineurin upon deimination.

**Metal ion dependency of deiminated calcineurin**

Calcineurin can be activated in vivo by a number of divalent metal ions, the most efficient among these being Mn$^{2+}$ and Ni$^{2+}$ (37,38). As stated before, the loss of calcineurin's phosphatase activity upon deimination was seen when calcineurin was assayed in the presence of Mn$^{2+}$ using pNPP as substrate. However, when Ni$^{2+}$ (0.5 mM) was used to activate calcineurin which had been incubated with deiminase and shown to have reduced activity when assayed with 1 mM MnCl$_2$ (control, 9.17 ± 0.74 nmol P$_i$/min, vs. deiminated, 3.96 ± 0.23 nmol P$_i$/min), the loss of activity was not observed (control, 3.48 ± 1.06 nmol P$_i$/min, vs. deiminated, 3.67 ± 0.29 nmol P$_i$/min). The reason for this result is unclear at this point, but may be related to different conformations the Mn$^{2+}$- and Ni$^{2+}$-stimulated enzymes may undertake upon metal binding (39). Indeed, the observation that deimination of the C-terminus of CaN A affects Ni$^{2+}$- and Mn$^{2+}$-activated calcineurin differently is consistent with the report that the autoinhibitory domain of CaN A influences Ni$^{2+}$ and Mn$^{2+}$ activation of calcineurin differently (40).
Figure 6. Fluorescence titrations of dansyl-calmodulin with calcineurin and deiminated calcineurin. Aliquots of control CaN (■) or deiminated CaN (●) were added to dansyl-calmodulin and increases in fluorescence intensity were measured 60 seconds after addition of titrant using a SPEX fluorolog 2 spectrophotometer. Inset: Hyperbolic plot of fraction CaM bound vs. free CaN (nM), with theoretical curves, as determined by the Enzfit software, superimposed upon the data.
Discussion

We have generated a modified form of calcineurin containing three citrulline residues localized to the 14 kDa C-terminal region of the CaN A subunit, which has previously been shown to contain the calmodulin binding domain of calcineurin (19). Conversion of these arginines to citrulline results in an enzyme with a significantly lower binding affinity for calmodulin and reduced activity when assayed under subsaturating calmodulin conditions. However, conversion of all three modified arginines is probably not required for these observed effects since addition of calmodulin to the deimination reaction yields a deiminated enzyme which retains full activity. This enzyme only contains an average of 1.55 mol citrulline/ mol calcineurin; therefore, modification of only one or two of the three modified arginine residues in CaN A may be necessary for the loss of activity upon its deimination in the absence of calmodulin.

Many studies on the interaction between calmodulin and the enzymes which it activates have focused on the role of hydrophobic residues either in the enzyme (41, reviewed in ref. 42) or in CaM itself (43-46). X-ray crystal (43) and NMR solution (44) structures of calmodulin bound to a peptide of smooth muscle myosin light chain kinase (smMLCK) show that the binding of calmodulin to its target is probably dominated by van der Waals forces. However, the data presented in this paper show the importance of the positively charged side chain of arginine residues in calcineurin-calmodulin binding, since the
introduction of citrulline at these positions results in the loss of the positive charge but does not drastically alter the structure of the side chain. Although Meador et al. (43) observed that 80% of the contacts between calmodulin and the smMLCK peptide were of the van der Waals type, they also noted that all seven basic residues of the smMLCK peptide make salt bridges with CaM.

The calmodulin-binding sequence in calcineurin is a predicted basic amphipathic α-helix which contains five arginines (20). Since one or two relevant arginine residues in the CaN A subunit were found to be modified by the deiminase, the modified residue(s) responsible for the loss of calcineurin's calmodulin binding may lie in this calmodulin binding sequence. Indeed, using both mutational and X-ray crystallographic techniques (43,44,47-49), arginine residues have been shown to be important for many interactions between calmodulin and the calmodulin binding sequence from smooth muscle myosin light chain kinase (smMLCK), a sequence with which calcineurin shows strong homology in its own calmodulin binding domain. Although the mechanism of activation differs between smMLCK and calcineurin (50-52), some similarities still may exist for calmodulin binding to the two enzymes, since this strong homology between them does occur. If this is the case, then modification of arginines in this region would surely disrupt calmodulin binding such as observed with the deimination of calcineurin.
Before more in depth analysis of the possible roles of arginines in the calmodulin binding domain of calcineurin is attempted, full characterization of the modified residues must be completed. The clostripain digestion conditions in this study favored complete digestion of the 14 kDa C-terminus of CaN A, leaving only a 44 kDa N-terminal fragment for analysis for citrulline. However, digestion conditions may be altered so that different defined derivatives of CaN A may be generated (19). In a preliminary experiment, we have altered the digestion conditions and generated a 45-46 kDa fragment of calcineurin (data not shown). This citrulline-containing 45-46 kDa fragment may be the N-terminal catalytic fragment of CaN A plus some C-terminal residues, which would support the idea that one or two arginines in the CaM-binding domain of CaN A are being modified by the deiminase. Further characterization of this fragment is in progress.

**Contributions of authors:** Tatsuo Senshu provided the anti-modified citrulline antibody for the Western blots. All other experiments were conducted and the manuscript was written by Jennifer Imparl-Radosevich, under the direction of Donald Graves.

**Literature Cited**


STUDIES OF THE ROLE(S) OF ARGinine IN THE REGULATORY DOMAINS OF CALCINEURIN

A paper to be submitted for publication to Archives of Biochemistry and Biophysics

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Abstract

In this paper we describe the effects of converting certain arginine residues to citrulline on two recombinant calcineurins (CaN). The first is a full-length rat brain calcineurin, denoted CaN500, and the second is a truncated calcineurin in which previously identified autoinhibitory sequences on the CaN A subunit have been deleted (denoted CaN457). Deimination of CaN500 results in the conversion of approximately 4 arginines to citrulline and in the reduction of its phosphatase activity in our assay to 20-40% of its original activity within one hour. Including calmodulin in the deimination reaction protects CaN500 from this loss of activity. Cleaving the C-terminal domain, where the calmodulin binding site of calcineurin is located, from deiminated CaN500 reactivates the phosphatase activity to the level of digested control CaN500. All these observations had been described using bovine brain calcineurin and had been shown to be due to an apparent reduced affinity of calmodulin for deiminated calcineurin (24). On the other hand, deimination of truncated CaN457 results in the incorporation of 0.5 mol/mol less citrulline than CaN500 and no loss of phosphatase activity. The reduced amount of citrulline in CaN457 could be due to truncation of an arginine which is normally deiminated on the full-length CaN500. Therefore, deimination of an arginine not present in the truncated CaN457 may be responsible for the 60-80% loss of phosphatase activity and reduced calmodulin binding of deiminated full-length rat and bovine brain calcineurins.
Introduction

Calcineurin, a calmodulin stimulated phosphatase (1,2), is a heterodimeric enzyme composed of a large subunit, CaN A, Mr 58-61 kDa, and a small subunit, CaN B, Mr 15-19 kDa (3-5). The phosphatase activity of calcineurin is inhibited by a complex of the immunosuppressant drugs cyclosporin A or FK506 and their respective intracellular receptors (6-8). The CaN A subunit contains the catalytic site (9) and sole calmodulin binding domain (10), whereas CaN B has 35% sequence identity to calmodulin (11) and binds four calcium ions with micromolar affinity (10). Recently shown to have highly homologous secondary structure to calmodulin (12), CaN B is thought to play a regulatory role in conferring additional calcium sensitivity to the enzyme in vivo (13).

The domain organization of the CaN A subunit has been elucidated through the work of several groups. Limited proteolysis of calcineurin showed distinct CaN B-binding, CaM-binding, and autoinhibitory domains, along with a 44 kDa N-terminal catalytic core (14-17). The putative CaM-binding region was identified by sequence homology to other CaM-binding peptides (18), while the autoinhibitory domain was identified to be 40-50 residues C-terminal from the CaM-binding sequence by peptide inhibition (19) and mutagenesis (20) studies. It is thought that the autoinhibitory region of CaN A acts to inhibit the phosphatase activity of the catalytic core. Binding of calmodulin presumably relieves this inhibition by displacing the
inhibitory region from the catalytic domain. However, the autoinhibitory domain of calcineurin is apparently not a "pseudosubstrate" for the active site of CaN A, since the peptide corresponding to the sequence of the autoinhibitory region is noncompetitive with respect to substrate (19).

Most calmodulin binding domains are predicted to form basic amphiphilic α-helices (21). The putative calmodulin binding domain of calcineurin is also predicted to form such a helix and contains five arginine residues (18). Many studies on the interaction between calmodulin and the enzymes which it activates have focused on the role of hydrophobic residues in the calmodulin binding site of the enzyme (21,22). However, not much is known about the importance of electrostatic interactions in calmodulin-target binding. What is known is that all seven basic residues in peptide corresponding to the CaM-binding sequence of smooth muscle myosin light chain kinase (smMLCK) make salt bridges with residues in calmodulin (23).

Previously, using peptidylarginine deiminase, we had generated a form of bovine brain calcineurin in which three positively charged arginine residues had been converted to the neutral amino acid citrulline (24). These citrulline were localized to the 14 kDa C-terminal region of the CaN A subunit, on which the CaM binding site is located. Modification of these arginines resulted in reduction of calcineurin phosphatase activity in our assay apparently due to a marked decrease in calmodulin binding affinity to calcineurin. Our results
suggested that not all of the arginines converted were necessary for the effects seen when these modifications were introduced. Therefore we wanted to localize further the modification(s) relevant to these observed effects. In this paper, we describe the deimination of a truncated form of CaN A in which C-terminal sequences of the autoinhibitory region, but not the putative calmodulin-binding domain, have been deleted. We find evidence suggesting that the relevant citrulline modification lies not in the previously described putative calmodulin-binding domain, but in the deleted C-terminal region of the CaN A subunit.

Experimental Procedures

Materials All buffers, as well as EGTA and phenyl-Sepharose, were purchased from Sigma. All other chemicals were from Fisher. PVDF membrane and HRP-conjugated goat anti-rabbit IgG were from BioRad. Serum-free insect cell media, Sf21 cells, and 3,3'-Diaminobenzidine tetrachloride (DAB) were from Life Technologies, Inc (Gaithersburg, MD). Recombinant baculoviruses containing the cDNA's for rat brain CaN500, CaN457 truncation mutant, and CaN B have been described (20,25) and were kindly provided by Dr. Thomas Soderling (Vollum Institute, Oregon Health Sciences University). Rabbit anti-calcineurin antibody was a generous gift from Frank Rusnak (Mayo Clinic, MN). Antibody recognizing modified citrulline residues in proteins has been described previously (26). [γ-32P]-ATP was from ICN Pharmaceuticals (Costa Mesa, CA).
Enzymes and proteins  Calmodulin was purified from bovine brain using the method of Sharma and Wang (27) modified to include phenyl-Sepharose after DE-52 chromatography, according to Gopolakushna and Anderson (28). CaM-Sepharose was prepared by the method of Sharma et al. (29). Peptidylarginine deiminase was purified from frozen rabbit skeletal muscle, according to Takahara et al. (30). Clostripain was purchased from Sigma. Protein concentration was determined by the method of Bradford (31).

Expression and purification of recombinant calcineurins

The expression of baculoviruses containing CaN A and CaN B cDNA's in Sf9 cells in media containing serum has been described (20,25). Expression and purification of these calcineurins were carried out similarly, with the following modifications. Sf21 cells in serum-free suspension culture were infected with third-passage CaN A and CaN B viruses at a multiplicity of infection of 5 and 7, respectively. The cells were then cultured at an initial density of 3 x 10^6 cells/ml in 40 ml serum-free insect cell media. Cells were harvested between 90-100 hours postinfection by centrifugation at 1000 x g for 10 minutes and resuspended in 20 ml buffer A (25 mM Tris, pH 7.8, 3 mM MgSO4, 2 mM EGTA, 2 mM EDTA, 20 mM 2-mercaptoethanol, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 5 mg/L leupeptin, 5 mg/L aprotinin, and 200 mg/L benzamidine). The resuspended cells
were either stored at -70°C or processed immediately for purification.

To lyse the cells for calcineurin purification, the cells were sonicated 5 x 15 seconds at 45 second intervals using a Branson sonifier 450. The homogenate was centrifuged at 20,000 x g for 30 minutes at 4°C and the supernatant made 45% in ammonium sulfate. Proteins were stirred for 10 minutes after addition of ammonium sulfate, then left on ice for 50 minutes. Precipitated proteins were collected by centrifugation at 11,000 x g for 30 minutes. The pellet was resuspended in 4 ml EGTA buffer (25 mM Tris, pH 7.8, 3 mM MgSO₄, 0.5 mM DTT, 0.1 mM EGTA) plus 0.5 mM PMSF, 5 mg/L leupeptin, 5 mg/L aprotinin, and 200 mg/L benzamidine and combined with 30 ml CaM-Sepharose in the same buffer. Calcium chloride was added to a final concentration of 2 mM, and the slurry was stirred intermittently for 30 minutes. The CaM-Sepharose was then washed with the following buffers on a glass scinttered funnel: CaCl₂ buffer (25 mM Tris, pH 7.8, 3 mM MgSO₄, 0.5 mM DTT, 0.2 mM CaCl₂) containing 2 mM CaCl₂, 5 x 50 ml; CaCl₂ buffer alone, 5 x 50 ml; and CaCl₂ buffer containing 0.3 M NaCl, 5 x 50 ml. All above CaCl₂ buffers were supplemented with 0.5 mM PMSF and 200 mg/L benzamidine. Calcineurin was eluted from the washed CaM-Sepharose with EGTA buffer containing 0.5 mM EGTA, 0.3 M NaCl, 0.5 mM PMSF, and 200 mg/L benzamidine. The protein peak was concentrated with an Amicon PM-10 membrane, dialyzed overnight against EGTA buffer
containing 20% glycerol, aliquotted, and stored at -70°C until use.

Calcineurin assays
Preparation of $^{32}$P-labeled RCMM-lysozyme was carried out as described (32,33). Typical calcineurin assays were done in 25 mM MOPS, pH 7.0, 1 mM MnCl$_2$, and the indicated concentrations of calmodulin (0.75-10 µg/ml), with 18 µM $^{32}$P-RCMM-lysozyme as substrate and 3 µg/ml calcineurin, in a reaction volume of 60 µl. The reaction was terminated by addition of 100 µl ice-cold 20% TCA. Protein substrate was allowed to precipitate on ice for 60 minutes, then the reaction was centrifuged at 15000 x g for 5 minutes. Released $^{32}$-phosphate in the supernatant was counted in 10 ml scintillant.

Deimination of expressed calcineurins
Deimination of expressed calcineurins was performed essentially as described (24). Briefly, reactions were carried out for 60 minutes in 50 mM Hepes, pH 7.2, 5 mM CaCl$_2$, 5 mM DTT, 0.7-1.1 mg/ml calcineurin, and 40 µg/ml deiminase. The full length CaN$_{500}$ was assayed in 10 µg/ml CaM in a typical calcineurin assay, while truncated CaN$_{457}$ was assayed in 0.75, 1.5, 3.0, and 10 µg/ml CaM.

Sodium-dodecyl sulfate-polyacrylamide gel electrophoresis, electroblotting, and Western analysis for citrulline
Calcineurin samples were separated by SDS-PAGE (34) on a 12.5% polyacrylamide gel and electroblotted onto PVDF using 10 mM CAPS, pH 11.0, in 10% methanol, or onto nitrocellulose using 25
mM Tris, pH 8.3, and 192 mM glycine in 20 methanol (constant 300 mA for 1 h). The PVDF membrane was stained with 0.025% (w/v) Coomassie brilliant blue R250 in 50% methanol (v/v) for amino acid analysis of protein bands, while nitrocellulose membranes were processed for Western analysis using an anti-modified citrulline antibody as described (24,26). Detection of immunoreactive bands was performed using the DAB/H2O2 colorimetric method as described (24).

Amino Acid Analysis Protein bands (CaN500 and CaN457) separated by SDS-PAGE and electroblotted onto PVDF were excised from the membrane and subjected to amino acid analysis. The analysis was performed on an Applied Biosystems 420A amino acid analyzer by the Iowa State University Protein Facility. Quantitation of citrulline was determined from the citrulline plus ornithine peaks in the HPLC trace, because degradation of citrulline into ornithine occurs during the acid hydrolysis (35).

Results

Expression of CaN500 and CaN457 truncation mutants The sequence of the CaN A subunit, along with a truncation mutant engineered so that a stop codon was introduced at amino acid 457, is outlined in Figure 1. The truncation mutant contains the full putative calmodulin domain, as described by Kincaid and Martin (18). However, the autoinhibitory region, as determined
Figure 1. Rat brain calcineurin A subunit amino acid sequence deduced from cDNA. Residues singly underlined denote the putative calmodulin binding domain as defined by Kincaid and Martin (18). Residues doubly underlined denote the autoinhibitory domain as described by Hashimoto et al. (19). (*) marks the truncation point for CaN A457, while (**) is the natural stop codon for CaN A500. RR are the arginines that have been mutated to alanine in the double mutant described by Perrino et al. (20).
MSEP KAIDPK LSTTD RVVKA VFPFPPSHRLT AKEVFNDDGK PRVDILKAHL
MKEGRL EEVS ARIIITEGAS ILRQEKNLLD IDAPVTVC GD IHGQFDL MK
LF EVGGSPAN TRYFLG DVY DRGYFSIECV LYLWALKI LY PKTLFLGRG
HECRHLTEYP TFQIECKIKY SERVYDACMD AFDCMLLAAL MNQQFLCVHG
GLSPEINTLD DIRKLDRFKE PPA YGPMCDI LWS DPLEDFG NEKTQUEHFT
NTVRGCSYF Y SYPVCD FLQ HNNLLSILRA HEAQDAGYRM YRKSQTTGFP
SLITIFSAPN YLDVYNNKAA VLKYENVMN IRQFNCSPHP YWLPNFMDVF
TW SLPFVG EK VTE MLV N LN ICSDDELGSE EDGF DGA TAA ARKEVI RNK I
RAIGKMARVF SVL RE E ES V T LTKGLPTTG MLPSGVLS GG KQTLQSAIKG
FSPQHK*ITSF EEAKG LDR IN E RMPPRRDAM PSDANL NSIN KALASE TNGT
DSNGSNSSNI Q**
by peptide inhibition studies (19) and mutagenesis (20), has been deleted. The only arginines which still exist in the truncation mutant are those of the catalytic core and the five arginines in the putative calmodulin-binding domain.

Since the CaN B subunit has been shown to be important for full activation of the phosphatase activity of the CaN A subunit, co-expression of CaN A and CaN B viruses was carried out using the baculovirus expression system. At first, we attempted to monitor calcineurin expression using an α-CaN polyclonal antibody. However, although the antibody recognizes purified bovine brain calcineurin, the antibody did not react to the expressed rat calcineurin (data not shown). We were able to show expression of calcineurin by SDS-PAGE of the homogenates of Sf21 cells infected with CaN A and CaN B viruses (Fig. 2). After CaM-Sepharose affinity chromatography, calcineurin was purified to 90-95% homogeneity, as judged by SDS-PAGE (Fig. 2). The expressed CaN B doublet in Figure 2 is a result of the myristylated (faster migrating) and non-myristylated (slower migrating) forms of the CaN B subunit, as shown previously (20).

Calmodulin dependence of expressed calcineurins The truncated form of calcineurin, CaN457, had previously been found to exhibit greater phosphatase activity than wild type calcineurin in the presence and absence of calmodulin (20), consistent with the deletion of the autoinhibitory region. However, CaN457 still is activated by increasing concentrations of calmodulin (Fig. 3), presumably because the enzyme still
Figure 2. (A) Coomassie-stained 12.5% SDS-PAGE of Sf21 cell homogenates expressing the CaN A500/CaN B or CaN A457/CaN B recombinant baculoviruses. Sf21 insect cells were made to express CaN A500 or CaNA457, along with CaN B, harvested 90-100 p.i., and resuspended in buffer A, as described in the Experimental Procedures. Cells were sonicated and 40 µl of each homogenate was loaded onto each lane. Lane 1, CaN A500/CaN B homogenate; Lane 2, CaN A457/CaN B homogenate. The CaN B doublet could not be accurately visualized in CaN-expressing homogenates due to co-migrating bands in non-expressing control homogenates (not shown). (B) Coomassie-stained 10% SDS-PAGE of calcineurins purified from baculovirus expression system. CaN500 (lane 2) and CaNA457 (lane 3) were purified using CaM-Sepharose affinity chromatography as described in the Experimental Procedures. Lane 1: molecular weight markers, 97, 66, 45, 31, 22, 14 kDa. 10 µg total protein was loaded onto each lane.
A.

1 2

CaN A → CaN A457
B.

(figure 2 continued)
Figure 3. Calmodulin activation curves of CaN<sub>500</sub> and CaN<sub>457</sub>. CaN<sub>500</sub> (■) and CaN<sub>457</sub> (○) were assayed as described in the Experimental Procedures, with increasing calmodulin concentrations in the assays.
contains the calmodulin binding site and sequences just N-terminal to the identified autoinhibitory region have recently been shown to have autoinhibitory properties (20). As expected, the phosphatase activity of CaN457 is greater than that of full-length CaN500 in the presence and absence of calmodulin. CaN457 can also be activated to the same level as CaN500 by slightly lower calmodulin concentrations, since $K_{act}$ of CaN457 by calmodulin is $1.1 \pm 0.1 \mu g/ml \text{CaM}$, while $K_{act}$ of CaN500 by calmodulin is $2.7 \pm 0.8 \mu g/ml \text{CaM}$. Therefore, lower calmodulin concentrations were used in calcineurin assays for CaN457 to attempt to detect decreased activity due to incubation of CaN457 with the deiminase, as described in the Experimental Procedures and also in the next section.

**Deimination of expressed calcineurins** When bovine brain calcineurin is incubated with peptidylarginine deiminase, its $\text{Mn}^{2+}/\text{CaM}$-stimulated phosphatase activity is reduced to 20-40% of the original activity within one hour (24). This was also observed with the full-length rat calcineurin expressed in S21 cells (Fig. 4A). Including calmodulin in the deimination reaction protects full-length calcineurin from this loss of activity (Fig. 5), as seen with bovine brain calcineurin (24), and even results in a slight activation of the enzyme. However, when the truncated CaN457 is incubated with the deiminase in the absence of calmodulin, no reduction in activity is observed after 90 minutes (Fig. 4B).
Figure 4. Time courses of incubation of CaN500 and CaN457 with deiminase. CaN500 or CaN457, at 0.9 mg/ml, was incubated with the deiminase as described in the Experimental Procedures. (A) Time course of CaN500 control (■) and CaN500 incubated with deiminase (●). (B) Time course of CaN457 control (□) and CaN457 incubated with deiminase (○). In this particular experiment, CaN457 was assayed in 3 μg/ml CaM. Other attempts at detecting a reduction in CaN457 activity using differing amounts of calmodulin in the assay (0.75, 1.5, and 10 μg/ml CaM) gave similar results.
B.

(figure 4 continued)
Figure 5. Calmodulin protection of the reduction in phosphatase activity of CaN$\textsubscript{500}$ during incubation with deiminase. The deimination reaction was performed as in Figure 4, except that calmodulin (42 μM, 3.7-fold molar excess over CaN$\textsubscript{500}$) was included in the reaction as indicated, then assayed.
When both of these calcineurins were assayed before and after deimination, different concentrations of calmodulin were used in the assay to keep both reactions within the linear range. A concentration of 10 µg/ml calmodulin was used for assaying the full-length CaN500, while 0.75, 1.5, 3.0, and 10.0 µg/ml calmodulin was used for the CaN457 assay. Note that the CaN500 assay contained almost saturating calmodulin, while most CaN457 assays were performed in subsaturating calmodulin (Fig. 3). The loss of activity of deiminated bovine brain calcineurin was previously observed under saturating calmodulin conditions, and a very high concentration of calmodulin was required to activate deiminated bovine brain calcineurin (24). Since loss of activity upon deimination of bovine brain calcineurin was observed under saturating calmodulin conditions, if loss of activity upon deimination of CaN457 was to be detected, it certainly would be observed under subsaturating calmodulin conditions.

**Localization of relevant modified arginines in calcineurin**

Deimination of full-length rat brain calcineurin, like bovine brain calcineurin, results in deimination of arginine residues only in the CaN A subunit (Fig. 6, lane 1). Unlike bovine brain calcineurin, proteolysis of the rat brain CaN500 subunit by clostripain, which results in the loss of the 14 kDa C-terminal region of CaN500, does not cleave all citrulline residues from the 44 kDa catalytic core (Fig. 6, lane 2). Although the
Figure 6. Western analysis of deiminated CaN500 and CaN457 using an anti-citrulline antibody. 5 µg deiminated CaN500 (lanes 1 and 2) or 5 µg deiminated CaN457 were loaded onto a 12.5% SDS-PAGE gel. Lanes 2 and 4 are CaN500 and CaN457, respectively, treated with 2 U clostripain as described (24). After electrophoresis, the separated subunits and proteolytic fragments were electroblotted onto nitrocellulose and analyzed for the presence of citrulline using an anti-citrulline antibody (see Experimental Procedures). No citrulline was observed in the CaN B subunit of either expressed CaN, while citrulline was present in the 61 kDa CaN A500 subunit (lane 1) and in the 57 kDa CaN A457 subunit (lane 3). The lower bands in the doublets in lanes 2 and 4 (both digested CaN's) correspond to the previously observed 44 kDa catalytic core (24). The slower migrating bands in the doublets presumably originate from each respective CaN A subunit and most probably represents a digested CaN A intermediate in the proteolysis reaction.
catalytic core appears to still contain citrulline, proteolysis of the C-terminal domain from the catalytic core relieves the inactivation caused by deimination to the level of control digested rat brain calcineurin (Fig. 7), an observation seen with bovine brain calcineurin (24). Therefore, another modification of an arginine(s) irrelevant to the reduction in activity upon deimination seems to be taking place in the catalytic core of the expressed rat brain calcineurin.

Amino acid analysis of full-length rat brain calcineurin shows an average of 3.85 citrulline residues in the CaN500 subunit, compared to an average of 2.95 citrulline in bovine brain. The three citrulline in the bovine brain calcineurin were localized to the 14 kDa C-terminal region of CaN A, with none in the catalytic core, so the extra citrulline found in the rat brain calcineurin may be due to modification of an arginine in the N-terminal catalytic core which is not modified in bovine brain. This is possible since sequence variations exist between the catalytic cores of bovine and rat brain calcineurins, especially in the extreme N-terminal region, which may be accessible to the deiminase (25,36, Fig. 8).

Western analysis of the truncated CaN457 also reveals that only the CaN A457 subunit of the truncated enzyme is deiminated (Fig. 6, lane 3). Upon proteolysis with clostripain, the 44 kDa fragment also contains citrulline, suggesting that the same modification(s) occurring in the N-terminal core of full-length
Figure 7. Reactivation of CaN500 upon limited proteolysis with clostripain. Digestion of control and deiminated full-length CaN500 was performed as previously described (24), then assayed. The closed bars show the activity of undigested control and deiminated CaN500 in the presence of 10 μg/ml calmodulin. The open bars show the activity of digested control and deiminated CaN500 in the presence of 10 μg/ml calmodulin.
Figure 8. N-terminal sequence comparison of bovine and rat brain calcineurins. N-terminal sequences of bovine and rat brain calcineurin differ so that recognition of arginines by the deiminase may also differ. Peptidylarginine deiminase appears to prefer certain arginines over others, depending on the sequence surrounding the arginines; however, no strict sequence specificity can be defined (35). N-terminal sequences are from references 25 and 36.
bovine  MAAPEPARAA PPPPPPPPPP PGADRVKAV PFPSSHRLTA...
rat    M SEPKAIDPKL STTDRVVKAV PFPSSHRLTA...
calcineurin is also taking place in the truncated form of calcineurin (Fig. 6, lane 4). The 44 kDa band from CaN457 is not as intense as the 44 kDa band from CaN500, which suggests that the truncated calcineurin may be more susceptible to protease treatment. Amino acid analysis of CaN457 shows an average of 3.35 citrulline/mol CaN457, 0.5 lower than full-length CaN500. This suggests that possibly one arginine in the region deleted in CaN457 is being modified in the full-length form of calcineurin, since the amount of citrulline is decreased in CaN457. Because no loss of activity is observed when CaN457 is incubated with the deiminase, an arginine that is deleted in the truncated form may be responsible for the loss of activity and reduced calmodulin binding of full-length calcineurin upon deimination.

Discussion

We have studied the effects of deimination on two recombinant calcineurins, one with a full-length CaN A subunit and the other with a truncated CaN A subunit in which certain autoinhibitory sequences have been deleted. Deimination of the full length calcineurin results in the reduction of its phosphatase activity to 20-40% of its original activity, as observed in the deimination of purified bovine brain calcineurin (24). Cleaving the C-terminal domain from the deiminated CaN500 using clostripain reactivates the phosphatase activity to the level of digested control CaN500. This suggests that modification of an arginine(s) in this C-terminal domain is
responsible for the loss of activity upon deimination. The observed loss of activity has been previously shown to be due to reduced calmodulin activation of and binding to calcineurin, using purified bovine brain calcineurin (24).

Unlike the full-length rat brain CaN500, the deiminated truncated CaN457 does not display reduced activity, even in the presence of several different subsaturating calmodulin concentrations. There could be several explanations for this observation. Perhaps the most interesting explanation is that the relevant arginine modification is taking place on CaN500 in the so-called autoinhibitory domain instead of the putative calmodulin-binding domain. This modification in the autoinhibitory domain could be affecting CaM-binding, thus the reduction in CaM-stimulated activity upon deimination of full-length CaN500. This would suggest that sequences in both the previously characterized calmodulin binding site and autoinhibitory region of calcineurin are important for complete CaM-binding and activation of calcineurin, when both regions are present. When both regions are not present, as in truncated CaN457, calmodulin can bind and presumably activate calcineurin's phosphatase activity to a greater degree for two possible reasons: (1) the additional constraint of calmodulin having to bind to additional C-terminal sequences would be gone and (2) a portion of the autoinhibitory domain is not binding to the catalytic core. Indeed, assaying deiminated CaN457 in the presence of several calmodulin concentrations suggests that CaM-
activation of CaN457 is not affected by deimination of residues outside of the deleted autoinhibitory region. On the other hand, calmodulin binding, and, in turn, calmodulin activation of purified bovine brain calcineurin is affected by deimination. There is precedence for calmodulin binding to two distinct sites in an enzyme; in phosphorylase kinase catalytic subunit, the C-terminal region, on which the autoinhibitory domain is located, contains two distinct calmodulin binding sites which bind calmodulin simultaneously (37).

Additional evidence that the arginine modification relevant to the loss of activity and presumed reduction in CaM binding is in the autoinhibitory region of CaN A is the amino acid analysis of both deiminated forms of calcineurin described in this paper. The full-length CaN500 contains 3.85 citrulline residues, while the citrulline is decreased to 3.35 in the truncated form. If the assumption is made that the same modifications are taking place in the catalytic core and/or previously identified CaM binding regions of both full-length and truncated calcineurin, then the reduced amount of citrulline in truncated CaN457 could be due to the deletion of the autoinhibitory region. Since, in the 14 kDa C-terminus of rat brain CaN A, no other arginine residues outside of the putative calmodulin binding or autoinhibitory domains exist, the decreased amount of citrulline in the truncated CaN457 might mean that the additional arginine
being modified in the full-length rat brain calcineurin is in the autoinhibitory region of CaN500.

A second explanation for no observed loss of activity upon deimination of CaN457 is that modification of a relevant arginine is still occuring in the putative CaM-binding domain of truncated CaN457 upon deimination, but loss of activity is not seen because additional secondary structure or sequences necessary for calmodulin activation or the observed loss of activity are not present. Another interpretation of the results could be that modification of an arginine to citrulline in the autoinhibitory domain causes an alternate or distorted secondary structure of the C-terminus of CaN A, resulting in decreased calmodulin binding and/or activation. In CaN457, this would not be possible since the autoinhibitory domain is deleted.

Finally, modification of an arginine in the autoinhibitory domain to citrulline could cause an increase in the affinity for its binding to the catalytic core. Again, this would not be possible in CaN457, for this region is deleted.

A double arginine mutant in the autoinhibitory region of CaN A has already been described (20, see also Fig. 1). In this mutant, the Ca²⁺/CaM-stimulated activity is activated 2-fold, as compared to the Ca²⁺/CaM-dependent activity of the wild type, while the phosphatase activity of the mutant in the presence of Ca²⁺ only (no CaM) is 3-fold that of Ca²⁺-stimulated wild type. The Ca²⁺ only activation of the mutant can be explained; the activation is probably due to decreased binding of the
autoinhibitory domain to the catalytic core upon mutation of these arginines (20). However, interpretation of the data is difficult as it pertains to CaM binding and activation of calcineurin, since those calcineurin assays were performed in saturating calmodulin. How well calmodulin binds to or activates the mutant is not clear. Therefore, single site-directed arginine mutations should provide more information to determine which arginine(s) are important for CaM-dependent loss of activity and binding to calcineurin upon its deimination. This information would tell us if these arginine(s) are located in the putative calmodulin binding domain, autoinhibitory domain, or both.

Contributions of authors: Tatsuo Senshu provided the anti-modified citrulline antibody for the Western blots. All other experiments were conducted and the manuscript was written by Jennifer Imparl-Radosevich, under the direction of Donald Graves.

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REGULATION OF PTP-1 AND INSULIN RECEPTOR KINASE BY FRACTIONS FROM CINNAMON: IMPLICATIONS FOR CINNAMON REGULATION OF INSULIN SIGNALLING

A paper to be submitted for publication

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Abstract

We have separated and identified two components derived from cinnamon, the "yellow" and "void" fractions, both of which inhibit PTP-1 activity and increase insulin receptor kinase catalytic autophosphorylation. The kinetic analysis of PTP-1 inhibition by the void fraction shows that it exhibits a time-dependent, irreversible inactivation of PTP-1 and is noncompetitive with respect to substrate, while the yellow peak
exhibits no such time-dependent inactivation of PTP-1. These fractions give high biological response, as measured by glucose oxidation in the rat epididymal fat cell assay. Wortmannin, a potent PI 3'-kinase inhibitor, decreases the biological responses to insulin and the mixture of these fractions (denoted 109301F) similarly, indicating that the cinnamon fraction is affecting an element(s) upstream of PI 3'-kinase. All these data may suggest that, in vivo, these fractions derived from cinnamon may be acting to stimulate insulin receptor kinase activity while it inhibiting PTPases which attenuate the receptor kinase signal. We also have partially characterized the yellow fraction, and mass spectrometry of this fraction shows that it contains a compound with a molecular weight of 346.

Introduction

Severe obesity or non-insulin dependent diabetes mellitus (NIDDM) is characterized by the diminished ability of insulin-dependent tissues to respond to insulin (1). This insulin-resistance could be the result of a number of defects in the insulin signalling pathway (2-6). However, much focus has been put on evidence that insulin receptor kinase autophosphorylation and subsequent phosphorylation of its principal substrate, IRS-1, are markedly decreased in insulin-responsive tissues of subjects with severe obesity or NIDDM (7-9). Dephosphorylation of the receptor β-subunit is associated with the deactivation of its kinase activity and therefore is associated with insulin
signal down-regulation (10). Recently, Nadiv et al. (11) have shown that the decreased degree of phosphorylation of the insulin receptor in the insulin-resistant condition may be due to the action of protein tyrosine phosphatases. The authors observed elevated cytosolic PTPase activity directed against the β-subunit of the insulin receptor in aged, insulin-resistant rats. Begum et al. (12) also reported increased PTPase activity toward intact insulin receptors in diabetic rat adipocytes, although they found the elevated PTPase activity in the particulate fraction. Regardless of the subcellular localization of the PTPases involved, enhancement of PTPase activity toward the insulin receptor kinase in insulin-dependent tissues apparently results in impaired insulin receptor signalling in those tissues.

One of the prime candidate PTPases likely responsible for dephosphorylation of the insulin receptor in vivo is PTP-1B. PTP-1B preferentially dephosphorylates a phosphopeptide encompassing the autophosphorylation domain of the insulin receptor β-subunit on Tyr-1150 or Tyr-1151 (13), sites critical for deactivation of the receptor kinase (10). When PTP-1B and insulin receptors were co-expressed to high levels, insulin receptor autophosphorylation was inhibited (14). Even PTP-1B mRNA levels are responsive to insulin. Treatment of rat hematoma cells with 100 nM insulin increases the abundance of the two mRNA's encoding PTP-1B by 1.6- and 3.1-fold, with no significant effect on the abundance of mRNA encoding the
receptor-type phosphatases LAR or LRP (15). Therefore, PTP-1B may provide feedback regulation of insulin signalling.

The development of oral anti-diabetic agents which enhance insulin sensitivity in insulin-dependent tissues has received much attention (16-18). One anti-diabetic agent, pioglitazone, has been shown to increase insulin sensitivity through its ability to reactivate insulin receptor kinase autophosphorylation (19,20). Pioglitazone does not act directly on the receptor, since incubation of insulin receptors and pioglitazone in vitro does not result in increased receptor kinase autophosphorylation (19). There is now strong evidence that this indirect effect of pioglitazone on receptor autophosphorylation is due, at least in part, to normalization of elevated PTPase activities in insulin-unresponsive cells (21). Preliminary evidence suggests that the cytosolic PTPase responsible for the normalization of elevated PTPase is PTP-1B (21). Therefore, some anti-diabetic agents may act to decrease elevated PTPase activities in insulin-dependent tissues, restoring insulin sensitivity to those tissues by allowing autophosphorylation of the insulin receptor and resultant insulin signal transduction.

Chromium has been shown to be required for maintaining normal glucose tolerance (22-24). Therefore, a slight chromium deficiency in humans could mean an increased risk of developing diabetes (25). However, there is mounting evidence that the chromium responsible for glucose tolerance maintainance is not
that of a simple inorganic chromium, such as chromium chloride, but that the chromium must be complexed with certain ligands to exert its biological activity (25-27). Two known biological chromium complexes have received much attention. The first, termed glucose tolerance factor (GTF), was originally extracted from Brewer's yeast and was found to contain chromium, nicotinic acid, glycine, glutamic acid, and cysteine (28). The second is known as low-molecular weight chromium binding ligand (LMWCr) (29). Having a molecular weight of ~1500, LMWCr contains chromium, aspartic acid, glutamic acid, and cysteine, but no nicotinic acid. LMWCr has been shown to exhibit biological activity, as detected by glucose oxidation in isolated fat cells, similarly to that of GTF (29).

Some extracts of certain spices also exhibit insulin potentiating activity in the rat epididymal fat cell assay (30-31). One of the spices which best potentiates insulin's effects in these cells is cinnamon (30). It is not known how cinnamon exerts its effect. It apparently is not due to the action of biological chromium in the spice, since chromium concentration in spices is not correlated with their insulin potentiating activity (30). A number of phenols, aldehydes, and acids have been identified in extracts of cinnamon, including eugenol, cinnamaldehyde, and cinnamic acid (32). It remains to be seen if any of these identified compounds are responsible for cinnamon's biological activity, or if a previously unidentified compound(s) is responsible.
The purpose of this work was to characterize a possible mechanism(s) by which cinnamon potentiates insulin's activity. A fraction from cinnamon, denoted 109301F, exhibits high biological activity in the rat epididymal fat cell assay. We tested the hypothesis that this fraction may potentiate insulin's effects by inhibiting a PTPase which dephosphorylates the insulin receptor kinase. We find that the fraction does indeed inhibit PTP-1, a rat homolog of PTP-1B, and that the inhibition is not likely due to the action of contaminating chromium. Surprisingly, the cinnamon fraction 109301F also activates insulin receptor catalytic domain autophosphorylation. This may indicate that 109301F is acting to stimulate insulin receptor kinase while it inhibits a PTPase which may otherwise attenuate the stimulated kinase activity. We further describe the separation of 109301F into two components which exhibit receptor kinase activation and PTP-1 inhibitory properties, as well as biological activity in the rat fat cell assay. One of the two components appears to have molecular weight of 346, as determined by mass spectrometry.

Experimental Procedures

Materials All buffers, along with polyGluTyr (4:1), alkaline phosphatase, wortmannin, and pNPP, were purchased from Sigma. All other chemicals were from Fisher. Serum-free insect cell media was from Life Technologies, Inc. (Gaithersburg, MD). [γ-32P]-ATP was from ICN Pharmaceuticals (Costa Mesa, CA).
Enzymes  Rat brain PTP-1 truncated at amino amino acid 323 to eliminate a hydrophobic region near the C-terminus was expressed as a GST-fusion and purified to homogeneity as described (33). The intracellular domain of the insulin receptor was expressed in the baculovirus expression system and purified as described (34), except that Sf21 cells instead of Sf9 cells were used for kinase expression and cells were harvested 96 h post infection. The expressed kinase domain contains the entire intracellular domain of the β-subunit of the human insulin receptor, but no sequences in the insulin-binding extracellular region. Therefore, kinase activity was insulin-independent and kinase activity was measured in the absence of insulin. Calcineurin was purified from Sf21 cells expressing the CaN A and CaN B subunits from rat brain essentially as described (35).

PTP-1 assays  

$^{32}$P-RCMM-lysozyme was prepared as described (36). For most assays, 20 µl enzyme diluted in assay buffer (50 mM Tris, pH 7.0, 0.05 mM EDTA, 1 mg/ml BSA, and 0.3% 2-mercaptoethanol) was added to 20 µl cinnamon compound or ddH$_2$O and 20 µl 300 nM $^{32}$P-RCMM-lysozyme, also diluted in assay buffer. Reaction was allowed to proceed so that less than 30% of the $^{32}$P was released from the $^{32}$P-RCMM-lysozyme (5-20 minutes, depending on enzyme dilution), then terminated by addition of 100 µl ice-cold 20% TCA. After 10 minutes on ice, the reaction was centrifuged at 15000 x g for 2 minutes, and the
supernatant counted in 10 ml scintillant. $^{32}$P-RCM-lysozyme as substrate for PTP-1 in all experiments, except where indicated, in which case pNPP was utilized.

PTP-1 activity toward pNPP was determined in the above assay buffer with 10 mM pNPP in a final reaction volume of 30 ul. Reaction was terminated by the addition of 0.5 ml 0.2 N NaOH, and the resulting pNP was measured at 410 nm in a Beckman spectrophotometer.

**Insulin receptor kinase domain assays**

For autophosphorylation assays, 7.1 μg kinase domain (0.24 mg/ml final concentration of kinase) was added to 1 mM $[^\gamma-^{32}P]$-ATP in 50 mM Hepes, pH 7.4, 20 mM MgCl$_2$, and 4 mM MnCl$_2$, in the presence or absence of cinnamon compound. The reaction was terminated by spotting 15 μl of reaction mixture on Whatman P-81 paper. The papers were washed 2 x 15 minutes in 0.5% H$_3$PO$_4$ and 1 x 5 minutes in ethanol. The dried papers were counted in 10 ml scintillant. For determining kinase activity toward exogenous substrate, the concentration of insulin receptor kinase domain was reduced to 20 μg/ml, and 1 mg/ml polyGluTyr (4:1) was included in the assay. Reaction was terminated by spotting 15 μl of the reaction on Whatman 3MM paper. The papers were washed 3 x 30 minutes in 10% TCA/1% pyrophosphate and 1 x 10 minutes in ethanol. The dried papers were again counted in 10 ml scintillant.
Purification of compounds from cinnamon. 1 g cinnamon was extracted with 20 ml 0.1N NH₄OH, as described (31). The pH was adjusted to 2.0, and the precipitate discarded. The pH of the supernatant was readjusted with NH₄OH to pH 10.0 and run on a Sephadex G-100 column in the same buffer. The void volume was discarded and the biologically active peak from the column run on a Sephadex G-50 column. The void volume from the Sephadex G-50 column was again discarded, while the biologically active peak was retained (this fraction was denoted 109301F). The above procedures were performed twice, once with 0.1N NH₄OH only, and once with 0.1N NH₄OH plus 20 mM sulfite, pH 10.0, which seemed to stabilize the biological activity through the columns. The initial studies of the effect of cinnamon compound(s) were performed with 109301F, obtained from the first chromatography run with no sulfite (0.1N NH₄OH only). The fraction obtained from the second Sephadex G-50 column, run in 0.1 N NH₄OH plus sulfite, and denoted 109301F-B, was further purified through a Sephadex G-25 column run in ddH₂O to obtain the "yellow" and "void" fractions.

Rat epididymal fat cell assay  Adipocyte isolation from rat epididymal fat pads was carried out as described (31). Glucose oxidation measurements of these adipocytes were also performed as described (31). Briefly, 0.43 µCi [U-¹⁴C]-glucose, 72 µg glucose and adipocytes were incubated with insulin or cinnamon fraction in a final reaction volume of 2 ml of Krebs-
Ringer phosphate buffer, pH 7.1. After quantitation of $^{14}$CO$_2$ release of the cells in response to insulin or cinnamon fraction, an insulin activity ratio was determined. The insulin activity ratio was calculated by dividing the cpm of $^{14}$CO$_2$ released by the cells in the presence of insulin or cinnamon fraction by that of fat cells alone. Control cells not treated with insulin or cinnamon exhibit an insulin activity ratio of one, which indicates an absence of biological activity (31).

**Mass spectrometry**   Electron impact and chemical ionization mass spectra were performed on a TSQ700 Finnigan MAT (San Jose, CA) mass spectrometer using Q1 only as a mass analyzer at the Instrument Services Laboratories at Iowa State University. Electron energy and current emission were maintained at 70 eV and 400 μA, respectively, while the source temperature was maintained at 150°C. Samples were introduced via a solids probe. Chemical ionization experiments were conducted using ammonia gas as reagent gas. Odd electron molecular ions were observed in the electron impact mass spectra while, in chemical ionization mass spectra, protonated and ammonia adduct ions were observed.

**Results**

Survey of potential PTP-1 inhibitors   A series of gel filtration fractions from cinnamon extract, along with a series of chromium compounds, were tested for the ability to inhibit rat brain PTP-1. As shown in Figure 1, most of the chromium
Figure 1. Survey of potential PTP-1 inhibitors: chromium compounds and cinnamon fractions. PTP-1 activity was measured using $^{32}$P-RCMM-lysozyme as substrate (see Experimental Procedures) in the absence (C-control) or presence of chromium compound or cinnamon fraction. (1) chromium nicotinate, 2.5 mg/ml; (2) chromium-nicotinate-glycine-galactose-cysteine, 0.8 mg/ml; (3) chromium picolinate, 1.6 mg/ml; (4) chromium picolate, 3.3 mg/ml; (5) chromium chloride, 10 mM; (6) 59301A; (7) 109301F; (8) F5257; (9) KG; (10) HPBY. The concentrations of chromium compounds described are final concentrations in the assay. Those concentrations were chosen to give the greatest soluble concentration possible in the assay. The final dilution of each of the cinnamon fractions in the PTP-1 assay was 1:6.
The graph shows the percentage of PTP-1 activity across different treatment groups labeled as chromium compound and cinnamon fraction.

- C: Control
- 1, 2, 3, 4: Various chromium compound treatments
- 5: Cinnamon fraction treatment
compounds were not able to inhibit PTP-1 phosphatase activity. Although CrCl$_3$ was able to inhibit PTP-1, it has been previously shown not to be biologically active in the rat epididymal fat cell assay (26). Therefore, we did not consider its PTP-1 inhibitory properties to be of interest for these purposes. In contrast to the chromium compounds, all of the fractions from cinnamon were found to inhibit PTP-1, for a 1:6 dilution in the assay. We then wanted to characterize further and compare how well each fraction from cinnamon, plus the inhibitory chromium nicotinate compound, inhibited PTP-1. The inhibitory dilution dependence curves for these fractions are shown in Figure 2. It appears from these curves that cinnamon fraction 109301F shows greater inhibition of PTP-1 activity at higher dilutions than the other fractions, although it must be noted that the relative concentrations of the inhibitory substance(s) is unknown in all the fractions. Because of this apparent ability to inhibit PTP-1 activity and the fact that this fraction also potentiated insulin's biological activity in the epididymal fat cell assay better than any of the other fractions (data to be presented elsewhere) we chose to work with 109301F and attempt to characterize its role in insulin potentiation.

**Effect of 109301F on phosphatases other than PTP-1** We tested the effect of 109301F on the phosphatase activities of alkaline phosphatase (37) and the calmodulin-activated phosphatase calcineurin (38,39). 109301F does not inhibit these phosphatases (Table I), with no effect on alkaline phosphatase
Figure 2. Inhibition dilution dependence curves for inhibition of PTP-1 by chromium nicotinate and cinnamon fractions. PTP-1 activity was assayed in the presence of serial dilutions of the inhibitors determined from Fig. 1. (■) chromium nicotinate dilutions from 2.5 mg/ml stock; (●) 59301A; (▲) 109301F; (◆) KG; (□) F5257; (◇) HPBY.
Table I. Effect of cinnamon fraction 109301F on phosphatase activities of alkaline phosphatase and calcineurin. Notes. (A) Alkaline phosphatase activity was measured in the absence or presence of a final dilution of 1:50 109301F. Using 0.75 mM pNPP as substrate, alkaline phosphatase was assayed continuously by monitoring p-nitrophenol formation at 410 nm in a Beckman DU-7 spectrophotometer. Assays were done in 1.0 M Tris, pH 8.0, with 0.4 μg/ml alkaline phosphatase. (B) Calcineurin activity was measured in the absence or presence of a final dilution of 1:30 in the assay. Calcineurin was assayed using pNPP as substrate as described (46).

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<th>(A) alkaline phosphatase (μmol/min/mg)</th>
<th>(B) calcineurin (nmol/min/mg)</th>
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<tr>
<td>control</td>
<td>60.0 ± 4.9</td>
<td>39.5 ± 4.8</td>
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<tr>
<td>109301F</td>
<td>59.5 ± 2.8</td>
<td>74.5 ± 5.6</td>
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activity and activation of calcineurin activity. This indicates that 109301F may be a specific protein tyrosine phosphatase inhibitor.

Kinetics of inhibition and time dependent inactivation of PTP-1 by 109301F If 109301F acted to inhibit PTP-1 activity by binding to the active site, then one would expect that the inhibitory effect of 109301F could be competed out with substrate and show kinetics competitive with respect to substrate. Figure 3 shows that 109301F is instead noncompetitive with respect to substrate.

To examine the possibility that 109301F could cause a time dependent inactivation of PTP-1, the enzyme and 109301F were incubated together at 30°C and diluted out at different times during the course of the incubation. The diluted enzyme from the different time points was then assayed without inhibitor. The final concentration of 109301F in the assay was such that no inhibition of PTP-1 in a separate control was observed (data not shown). Figure 4 shows that a time-dependent, irreversible inactivation of PTP-1 indeed results upon incubation of PTP-1 with 109301F. A very fast inactivation of PTP-1 seems to occur within the first five minutes of incubation with 109301F, while a slower inactivation occurs through 30 minutes of incubation. This suggested that the inactivation of PTP-1 by 109301F may be due to more than one inhibitory substance in the fraction. These results also suggest that irreversible inactivation of the enzyme obfuscates the interpretation of the kinetics of Fig. 3.
Figure 3. Kinetics of inhibition of PTP-1 by 109301F. PTP-1 activity was measured using a range of 25–1000 nM $^{32}$P-RCMM-lysozyme in the absence of inhibitor (■) or in the presence of a final dilution of 1:100 (●) and 1:300 (▲) 109301F.
Figure 4. Time-dependent, irreversible inactivation of PTP-1 upon incubation with 109301F. PTP-1 and 109301F (●) were preincubated together at 30°C at 1:3500 and 1:150, respectively. (■) is the PTP-1 control preincubated in assay buffer only. At the indicated times, an aliquot of the preincubation mixtures were diluted 1:100 and placed on ice until the diluted enzyme was assayed. 20 μl of diluted enzyme was used in a typical PTP-1 assay (see Experimental Procedures). The final concentration of 109301F which carried over into the PTP-1 assay after dilution from the preincubation mixture was 1:45000. This concentration of 109301F did not inhibit freshly added PTP-1 which was not preincubated with 109301F (data not shown, see also Fig. 2).
Non-competitive inhibition normally might be interpreted to mean that binding of the inhibitor is occurring outside the active site region. However, if binding did occur in the active site and caused partial irreversible inactivation of the enzyme, the results of Fig. 3 would be expected.

To determine if this irreversible inactivation was taking place at the active site of PTP-1, arsenate, a competitive inhibitor of PTP-1 (K_i = 0.24 mM (47)), was included in the preincubation of PTP-1 with the void fraction. Addition of 1 mM arsenate during the preincubation results in only 71% inhibition of PTP-1 after 30 minutes of preincubation vs. 97% inhibition of PTP-1 without arsenate (data not shown). This partial protection of PTP-1 from inactivation by the void fraction in the presence of the competitive inhibitor arsenate may indicate that the inhibitor is acting at the active site of the enzyme.

Sephadex G-25 chromatography of 109301F-B Because the inactivation of PTP-1 by 109301F may have been due to more than one inhibitory substance (Fig. 4), an attempt was made to separate these possible components. 20 ml of the 109301F-B fraction from the second Sephadex G-50 chromatography run with sulfite was concentrated in a Savant speed vacuum to approximately 1 ml, then 0.5 ml of this concentrated fraction chromatographed on a 25 x 1 cm Sephadex G-25 gel filtration column. The resulting separated peaks in order of their elution are illustrated in Figure 5. They were labeled the "void", "red", and "yellow" peaks, because the orange-brown peak came
Figure 5. Sephadex G-25 chromatography of 109301F. The 109301F fraction in bisulfite buffer was concentrated in a Savant speed vacuum to approximately 1 ml. The fraction was then chromatographed on a 25 x 1 cm Sephadex G-25 gel filtration column, using ddH2O as buffer. The resulting separated peaks are shown in order of their elution.
void

red

yellow
out in the void volume and the other two peaks were red and yellow in color.

We were concerned that, since the red and yellow peaks eluted rather late in the Sephadex G-25 chromatography, these peaks could have co-eluted with the low molecular weight sulfite from the sulfite buffer. Therefore, we qualitatively tested this by adding H₂O₂ to oxidize any sulfite present to sulfate, then we added BaCl₂ to precipitate any sulfate present as BaSO₄. Using this test we found no sulfite present in the void fraction, as expected. However, the yellow and red fractions, having been concentrated 10-fold after elution from Sephadex G-25, contained <60 mM sulfite, based on spectral data obtained at the absorbance maximum of sulfite (A₂₇₆) with standard solutions of known sulfite concentrations (not shown). This estimation was also corroborated by comparison of the BaSO₄ precipitate to a known amount of sulfite (data not shown). Because of the limiting quantity of the red fraction, we decided to further characterize only the void fraction and cautiously continue characterization of the yellow fraction. We describe control experiments in which enzyme activities were measured in the presence of sulfite where appropriate in this text.

Inhibition of PTP-1 by the yellow fraction Both of the yellow and void peaks from the Sephadex G-25 chromatography inhibit PTP-1 (Table II) and are biologically active (data to be presented elsewhere). The inhibition of PTP-1 by the yellow fraction using ³²P-RCMM-lysozyme as substrate (Table IIA) is
Table II. Inhibition of PTP-1 by fractions from Sephadex G-25 chromatography. Notes. (A) PTP-1 activity was measured in the absence (control) or presence of a final dilution of 1:6 void or yellow fraction using $^{32}$P-RCMM-lysozyme as substrate. The final concentration of sulfite carried over from the yellow fraction was <10 mM sulfite. (B) PTP-1 activity was measured in the absence (control) or presence of a final dilution of yellow or void fraction as indicated, using 10 mM pNPP as substrate. The final concentration of sulfite carried over from the yellow fraction in the 1:12 and 1:120 dilutions was <5 mM and <0.5 mM, respectively.

(A) $^{32}$P-RCMM-lysozyme

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<tr>
<td>control</td>
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</tr>
<tr>
<td>1.0 mM sulfite</td>
</tr>
<tr>
<td>6.6 mM sulfite</td>
</tr>
<tr>
<td>1:6 void</td>
</tr>
<tr>
<td>1:6 yellow</td>
</tr>
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(B) pNPP

<table>
<thead>
<tr>
<th>% activity</th>
</tr>
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<tr>
<td>control</td>
</tr>
<tr>
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</tr>
<tr>
<td>10.0 mM sulfite</td>
</tr>
<tr>
<td>1:12 void</td>
</tr>
<tr>
<td>1:12 yellow</td>
</tr>
<tr>
<td>1:120 yellow</td>
</tr>
</tbody>
</table>

*no counts above background could be detected
complicated by the fact that the assay contains <10 mM sulfite carried over from the dilution of the yellow fraction. This is because only 6.6 mM sulfite in the assay inhibits the dephosphorylation of $^{32}$P-RCMM-lysozyme by PTP-1 by 90%. In contrast, when we used the low molecular weight substrate pNPP at the saturating concentration of 10 mM ($K_m = 3.5$ mM (36)), 10 mM sulfite was found not to inhibit PTP-1 (Table IIB). The yellow fraction at a final dilution of 1:12 and a contaminating concentration of sulfite of <5 mM inhibited PTP-1 activity by 85%. Therefore, the yellow fraction does contain at least one component which inhibits PTP-1, and this inhibition is not due to sulfite. This was not observed using $^{32}$P-RCMM-lysozyme as substrate, probably because saturating substrate was not used in these assays.

**Inhibitory dilution dependence curve for inhibition of PTP-1 by the void peak**

The orange-brown color of the void peak most closely resembles that of the 109301F fraction. Therefore, we wanted to determine if the inhibitory dilution dependence of the void peak was similar to that of the 109301F fraction. As shown in Figure 6, the dilution dependence of the PTP-1 inhibitory property of the void peak does closely resemble that of the 109301F fraction in Figure 2.

**Effect of preincubation of Sephadex G-25 peaks with PTP-1**

To determine which Sephadex G-25 peak was capable of inactivating PTP-1 in a time-dependent manner, the experiment in Figure 4 was repeated with the yellow and void fractions from
Figure 6. Inhibition dilution dependence curve for inhibition of PTP-1 by the void fraction.

Inhibition dilution dependence curve of inhibition of PTP-1 by the void fraction was determined as in Figure 2, using only the void fraction from Sephadex G-25 chromatography as inhibitor.
% PTP-1 activity vs log [final dilution of void peak in assay]
Sephadex G-25 chromatography. As shown in Figure 7, incubation of PTP-1 with only the void peak shows inactivation of PTP-1 in a time-dependent manner. Therefore, we conclude that at least one substance in 109301F that is responsible for the time-dependent inactivation of PTP-1 is in the Sephadex G-25 void fraction.

**Kinetics of inhibition of PTP-1 by Sephadex G-25 void peak**

Since it was apparent that some components in 109301F had been separated from each other during Sephadex G-25 chromatography, kinetic analysis of the void fraction was performed. The kinetics of void peak inhibition of PTP-1 shows noncompetitiveness with respect to substrate (Fig. 8). This again is reminiscent of the kinetics of the 109301F mixture, indicating that the noncompetitive kinetics exhibited by 109301F is probably due to the component(s) in the void peak.

**Effect of wortmannin on the insulin potentiating activity of cinnamon**

Wortmannin is an antifungal agent which inhibits PI 3'-kinase in the nanomolar range (40-41), which, in turn, inhibits many effects of insulin stimulation in insulin-dependent cells (42). To determine if the insulin potentiating effects of cinnamon were affected by wortmannin, rat adipocytes were incubated with different amounts of the agent and the insulin response was measured in the presence 109301F. Figure 9 shows that the biological potentiation in response to both insulin and cinnamon is dramatically reduced as the concentration of wortmannin is increased. This indicates that
Figure 7. Effect of preincubation of PTP-1 with yellow and void fractions. PTP-1, at a dilution of 1:3500, was incubated with the yellow (●) or void (○) fraction at a final dilution of 1:50 or 1:100, respectively. (■) is control PTP-1 preincubated in assay buffer only. At 2, 4, 6, 10, 15, and 30 minutes, aliquots of each of the preincubation mixtures were diluted 1:100 and assayed as in Figure 4. The final dilution of inhibitor (yellow or void) in the preincubation mixture was chosen so that PTP-1 inhibition was 35-50% in a separate assay with that final dilution in the PTP-1 assay without preincubation or dilution to 1:100.
Figure 8. Kinetics of inhibition of PTP-1 by the void peak from cinnamon. PTP-1 activity was measured using a range of 25-1000 nM $^{32}$P-RCMM-lysozyme in the absence of inhibitor (■) or in the presence of a final dilution of 1:100 (●) and 1:200 (▲) of the void fraction.
Figure 9. Wortmannin effects on adipocytes activated by cinnamon fraction 109301F or insulin. Fat cells were isolated as described (31) and incubated with the designated amounts of wortmannin. Wortmannin was dissolved in dimethylsulfoxide and 10 μl of DMSO containing wortmannin were added to each tube. The purified cinnamon fraction 109301F (25 μl, dry weight less than 1 mg/ml, open bars), or porcine insulin (100 μU/ml, closed bars) was added to tubes. Tubes contained 3.8 mg of total fat.
The graph shows the effect of wortmannin (nM) on potentiation.

Potentiation is measured on the y-axis, while wortmannin concentration (nM) is on the x-axis. The graph indicates a decrease in potentiation as the concentration of wortmannin increases.
the cinnamon fraction acts to signal through PI 3'-kinase in insulin-dependent cells.

Effect of different cinnamon fractions on insulin receptor kinase

Since inhibition of PI 3'-kinase inhibits cinnamon signalling, this suggests that the cinnamon fraction is acting upstream of PI 3'-kinase. The known upstream elements in activating PI 3'-kinase signalling in response to insulin are IRS-1 and the insulin receptor itself (43). Since we observed that 109301F inhibited PTP-1, which in vivo may result in the increased autophosphorylation of the insulin receptor kinase (13-15), we wanted to determine if 109301F had any direct effect on the catalytic autophosphorylation of the kinase. As shown in Figure 10A, 109301F directly activates the autophosphorylation of the insulin receptor kinase catalytic domain. However, this effect is transient, since preincubation of 109301F with the kinase for 30 minutes prior to addition of [γ-32P]-ATP results in basal level kinase autophosphorylation. The reason for this is unclear, but the activation is due to autophosphorylation on the kinase (Fig. 10B) and not to phosphorylation of the components in 109301F and those phosphorylated components binding to the P-81 paper in the assay. Therefore, since 109301F inhibits PTP-1, a protein tyrosine phosphatase that may act in vivo to regulate the activity of the insulin receptor kinase, and activates the insulin receptor kinase autophosphorylation directly, this cinnamon fraction may act in vivo to do the same. The net result would be increased
Figure 10. Effect of preincubation of insulin receptor kinase domain with 109301F on kinase autophosphorylation. 240 μg/ml receptor kinase domain was preincubated with a final dilution of 1:150 109301F for the indicated times, at which point \(^{32}\)P-ATP was added to start a 5 minute autophosphorylation reaction. (A) The autophosphorylation reaction was terminated by addition of EDTA to 45 mM and then 15 μl of the reaction mixture was spotted onto P-81 paper. Papers were washed and counted as described in the Experimental Procedures so that % incorporation of \(^{32}\)P into the kinase could be calculated. (B) 3.0 μl of 5X SDS gel loading buffer was added to 12 μl of the EDTA-terminated reactions from above and then heated at 100°C for 3 minutes. The samples were electrophoresed on a 12.5% SDS-PAGE gel, which was dried down and exposed to film for approximately 15 minutes.
B.

\[
\text{no } 109301F \quad + \quad 109301F
\begin{array}{c}
0 & 5 & 30 & 0 & 5 & 30
\end{array}
\]

(figure 10 continued)
autophosphorylation of the receptor kinase and presumably increased insulin signalling.

After the void and yellow peaks were obtained from Sephadex G-25 chromatography, the effect of these peaks on the insulin receptor kinase catalytic domain was tested. Figure 11 shows the time course of the insulin receptor kinase autophosphorylation reaction in the presence of a 1:30 dilution of each peak. Both of the peaks activate kinase autophosphorylation, although the void peak appears to activate the kinase better than the yellow peak. It again must be noted that the concentration of the component(s) of these peaks is unknown, so comparison of the relevant activities is difficult. Activation of the receptor kinase by the yellow fraction is apparently not due to contaminating sulfite (estimated at <2 mM in the assay), since 6.6 mM sulfite had no effect on kinase autophosphorylation (data not shown).

Since the autophosphorylation of the insulin receptor kinase is activated by these peaks from G-25 chromatography, we wanted to test the effect of the peaks on the kinase activity toward exogenous substrate. Unexpectedly, the insulin receptor kinase activity toward the exogenous substrate polyGluTyr (4:1) is inhibited in the presence of both G-25 peaks (Table III). However, it must be noted that the concentration of kinase in the polyGluTyr assay (20 μg/ml kinase) was such that no autophosphorylation of the kinase was possible. It is widely accepted that, in vivo, insulin acts to stimulate receptor
Figure 11. Time course of receptor kinase autophosphorylation in the presence of the yellow and void fractions from cinnamon. 240 μg/ml kinase domain was allowed to autophosphorylate in the absence of cinnamon peak (■) or in the presence of a final dilution of 1:30 the yellow (▲) or void (♦) fractions. At the times indicated, the autophosphorylation reactions were terminated by spotting 15 μl of each reaction onto P-81 paper. Papers were washed and counted as described in the Experimental Procedures.
Table III. Effect of yellow and void fractions on receptor kinase activity toward exogenous substrate. Notes. Kinase activity toward polyGluTyr (4:1) was measured as described in the Experimental Procedures in the presence of a final dilution of 1:30 yellow or void fraction.

<table>
<thead>
<tr>
<th>sample</th>
<th>pmol $^{32}$p/μg polyGluTyr</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>6.5 ± 0.3</td>
</tr>
<tr>
<td>void</td>
<td>0.55 ± 0.18</td>
</tr>
<tr>
<td>yellow</td>
<td>0.18 ± 0.06</td>
</tr>
</tbody>
</table>
kinase autophosphorylation, which, in turn, activates the kinase toward exogenous substrate. The cinnamon fraction 109301F does indeed increase autophosphorylation of the kinase, in assays where the concentration of kinase were such that autophosphorylation could take place (240 μg/ml kinase, in Figures 10 and 11). Therefore, if the role of 109301F in insulin signalling were to increase receptor autophosphorylation, then allowing the kinase domain to autophosphorylate first in the presence of the cinnamon fraction might result in increased activity of the kinase toward exogenous substrate.

To examine this possibility, we incubated the insulin receptor kinase domain (240 μg/ml) with one of the G-25 peaks, the yellow peak, and [γ-32P]-ATP so that increased autophosphorylation of the kinase could take place (Table IVA). Then, the autophosphorylated kinase was diluted (20 μg/ml) to measure its activity toward exogenous substrate, in the presence and absence of additional yellow peak. The results are shown in Table IVB. Although kinase activity toward polyGluTyr (4:1) in the absence of additional yellow peak was not increased, the kinase activity toward polyGluTyr (4:1) in the presence of yellow peak showed a 2.5-fold increase over that of kinase not allowed to autophosphorylate in the presence of yellow peak (Table IVB). Therefore, increased autophosphorylation in the presence of the cinnamon yellow peak does increase its activity toward exogenous substrate, when assayed in the presence of the
Table IV. The effect of previous autophosphorylation of insulin receptor kinase domain in the presence of the yellow fraction from cinnamon. Notes. (A) 240 µg/ml receptor kinase was allowed to autophosphorylate for 5 minutes in the absence or presence of a final dilution of 1:30 of the yellow fraction, as described in Fig. 10A. (B) The autophosphorylated samples from (A) were diluted into a polyGluTyr assay to a final concentration of 20 µg/ml kinase. "no yellow in assay" describes the kinase activity of both autophosphorylated samples (+ and - yellow fraction in the autophosphorylation reaction) toward polyGluTyr in the absence of additional yellow peak. "+ yellow in assay" describes the kinase activity of both autophosphorylated samples (+ and - yellow fraction in the autophosphorylation reaction) toward polyGluTyr with additional 1:30 yellow fraction included in the polyGluTyr assay.

<table>
<thead>
<tr>
<th></th>
<th>$^{32}$p incorporation</th>
<th>% incorporation (pmol $^{32}$p/µg polyGluTyr)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>control</td>
<td>20.5 ± 4.6</td>
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<tr>
<td></td>
<td>with yellow peak</td>
<td>41.9 ± 3.0</td>
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<tr>
<td>no yellow in assay</td>
<td>control</td>
<td>21.8 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>previous yellow</td>
<td>23.2 ± 1.1</td>
</tr>
<tr>
<td>+ yellow in assay</td>
<td>control</td>
<td>0.292 ± 0.009</td>
</tr>
<tr>
<td></td>
<td>previous yellow</td>
<td>0.692 ± 0.003</td>
</tr>
</tbody>
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yellow peak. It is not clear why this difference is not observed when kinase autophosphorylated in the presence of the yellow peak, but assayed with polyGluTyr (4:1) in the absence of this peak.

Partial chemical characterization and comparison of cinnamon peaks from Sephadex G-25 chromatography. Absorbance spectra of the void and yellow peaks were taken to make comparisons of these fractions. As shown in Figure 12, the void fraction gives an absorbance at 320 nm in high pH, but the peak shifts to 291 nm and decreases in intensity in low pH. In contrast, Figure 13 shows that the yellow fraction, at pH 6.3, gives a small absorbance at 305 and 276 nm, but as pH is lowered, the 305 nm peak is lost while the intensity of the 276 nm increases dramatically.

Because a sulfite control solution also exhibits an absorbance peak at 276 nm which increases in intensity upon lowering the pH, it may appear that the absorbance of the yellow fraction at 276 nm is due solely to sulfite. However, 5 mM sulfite at pH 6.1 does not exhibit detectable absorbance at 276 nm, while the absorbance of a dilution of 1:50 yellow fraction at pH 6.3 is 0.175 (not shown). The final concentration of sulfite in the diluted yellow fraction is only <1.33 mM. Therefore, some, but not all, of the absorbance at observed at 276 nm in the yellow fraction is due to contaminating sulfite.

The yellow fraction was then analyzed by both electron impact (EI) and chemical ionization (CI) mass spectrometry to
Figure 12. Absorbance spectra of void fraction in base and acid. All absorbance spectra were taken with a Beckman DU-7 spectrophotometer. Traces of void fraction in base are pH 11.2 and 12.0 (A_{320} peaks), while the traces in acid are pH 1.7, 1.9, and 2.2 (A_{291} peaks).
Figure 13. Absorbance spectra of the yellow fraction. An absorbance peak at 276 nm increased with decreasing pH. Traces, from the lowest A_{276} to highest A_{276}, are in pH 6.3, 3.2, 2.5, 2.3, 1.94.
determine the molecular weight of the compound. In electron impact mass spectrometry, the sample is bombarded by a stream of electrons, resulting in a molecular ion deficient in just one electron (M⁺'). EI analysis of the yellow fraction reveals a parent molecular ion of a molecular weight of 346 (Fig. 14A). We wanted to confirm this result, so the sample was also analyzed by chemical ionization mass spectrometry. In CI, the sample is ionized in the presence of reagent NH₃ gas, so both ammonium adduct (MNH₄⁺) and protonated (MH⁺) parent molecular ions should be observed. This is indeed the case (Fig. 14B), with the ammonium adduct ion having a molecular weight of 364 (346 + NH₄⁺) and the protonated ion having a molecular weight of 347 (346 + H⁺). Therefore, a substance in the yellow fraction appears to have a molecular weight of 346.

Discussion

We have found that the cinnamon fraction 109301F does inhibit PTP-1 activity, and that the PTP-1 inhibition is probably not due to the action of contaminating chromium. This is in accordance with the finding that chromium content in spices is not correlated with their insulin potentiating activity (30). The fraction 109301F, later found to be a mixture of components, also activates insulin receptor catalytic autophosphorylation.

We have separated and identified two components derived from the cinnamon fraction 109301F, the yellow and void components, both of which inhibit PTP-1 activity and increase
Figure 14. (A) Electron impact mass spectrometry of the yellow fraction. (B) Chemical ionization mass spectrometry of the yellow fraction.
(figure 14 continued)
insulin receptor kinase catalytic autophosphorylation. The mixture of these fractions (109301F) gives high biological response in the rat epidydimal fat cell assay. Wortmannin, a potent PI 3'-kinase inhibitor, decreases the biological responses to both cinnamon and insulin similarly, indicating that the cinnamon fraction is affecting elements upstream of PI 3'-kinase. All these data may suggest that, in vivo, this mixture is acting to stimulate insulin receptor kinase activity while it inhibits PTPases which may attenuate the receptor kinase signal.

The kinetic analysis of PTP-1 inhibition by the void fraction shows that it exhibits a time-dependent, irreversible inactivation of PTP-1 and is noncompetitive with respect to substrate. The irreversible inhibition of PTP-1 by the void may indicate that the compound(s) in the void fraction are covalently modifying the PTP-1. No shift in mobility on SDS-PAGE was observed after PTP-1 incubation with and inactivation by the void (data not shown). However, since the identity of the inhibitor in this case is not known, nothing can be inferred about the lability of a possible covalent linkage between the void and PTP-1 during electrophoresis and/or in the presence of SDS.

The yellow and void peaks both act to increase the autophosphorylation rate of the insulin receptor kinase, but this effect is transient. This is because preincubation of the kinase with the cinnamon fraction 109301F for 5 or 30 minutes
results in a kinase with a basal autophosphorylation rate. The yellow fraction also acts to increase receptor kinase catalytic activity toward exogenous substrate, but only if the kinase is allowed to first autophosphorylate in the presence of the yellow peak and then is diluted into the polyGluTyr assay in the presence of additional yellow peak. No increase in kinase exogenous substrate activity is observed if the polyGluTyr assay is performed in the absence of additional yellow peak, even if the kinase is allowed to first autophosphorylate in the presence of the yellow fraction. It is not clear why this is, but perhaps it is related to the transient effect of the cinnamon fraction 109301F on the kinase. As mentioned before, only a 5 minute preincubation of the kinase with 109301F results in only basal autophosphorylation of the kinase. In the experiment described in Table III, the kinase was allowed to autophosphorylate for 5 minutes in the presence and absence of the yellow fraction, then the enzyme was diluted for analysis of activity toward polyGluTyr. Perhaps addition of more yellow peak in the polyGluTyr assay would be the only way to observe a difference in activity, since, at that point in the experiment, the cinnamon fraction had already been incubated with the kinase for 5 minutes.

The transient nature of the cinnamon fraction 109301F on the kinase may indicate that a compound(s) in it may actually be reacting with the receptor kinase itself and, in turn, be increasing the kinase activity. However, this active cinnamon-
kinase complex seems to be unstable, since prolonged stimulation of the kinase is not observed upon preincubation of it with 109301F for 5 minutes. The instability of the complex may result in the dissociation of the kinase from the compound(s) and/or may change the complex so that it can no longer activate the kinase. The inactivation of the cinnamon fraction 109301F upon incubation is not likely due to the buffer components of the kinase assay. This is because pre-incubation of 109301F with kinase buffer for 30 minutes has no effect on its ability to activate receptor kinase autophosphorylation (data not shown).

Recently there has been a report of a specific compound which reacts with the β-subunit of the insulin receptor and acts to increase its basal kinase activity toward exogenous substrate. Bernier et al. (44) used maleimidodibutyrylbiocytin (MBB) to biotinylate sulfhydryls on the β-subunit of the human insulin receptor in permeabilized CHO/HIRC cells. The modified kinase exhibited a 2-fold increase in the basal kinase activity toward exogenous substrate. Perhaps the compound(s) from cinnamon is acting on the kinase similarly.

We attempted to partially characterize the two peaks from Sephadex G-25 chromatography. Electron impact and chemical ionization mass spectrometry of one of the fractions, the yellow fraction, show that it contains a compound with a molecular weight of 346. Further characterization of the chemical properties of this compound is in progress.
Acknowledgements

The authors would like to extend their gratitude to Debra Baedke and Thomas Ingebritsen for the purification of the expressed PTP-1 and insulin receptor kinase domain.

Contributions of authors: Marilyn Polansky conducted the initial cinnamon extractions and Sephadex G-100 and G-50 chromatographies, along with the rat epidydimal fat cell assays, under the direction of Richard Anderson. All other in vitro enzyme experiments were conducted and the manuscript was written by Jennifer Imparl-Radosevich, under the direction of Donald Graves.

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In this dissertation, aspects of regulation of two protein phosphatases involved in signal transduction are described. The first phosphatase, calcineurin, has been shown to be involved in T-cell activation, since inhibition of calcineurin by immunosuppressant treatment inhibits IL-2 production, and, in turn, T-cell activation (37,39). Calcineurin is also presumed to be regulated by Ca^{2+} in vivo because CaN B, the small subunit of calcineurin, is a Ca^{2+}-binding protein, and calmodulin, another Ca^{2+}-binding protein, binds to and activates calcineurin in a Ca^{2+}-dependent manner (1,5,6). Therefore, we sought to better understand this Ca^{2+}-activation of calcineurin by attempting to understand more about the mechanism of activation of calcineurin by calmodulin.

Because most studies on the interaction between calmodulin and the enzymes which it activates have focused on the role of hydrophobic residues in the interaction (56-58,65-67), we decided to look at the role of the positively charged arginine residue in calcineurin-calmodulin interaction. Certain arginine residues in the C-terminal portion of bovine brain CaN A were converted to the neutral amino acid citrulline by the action of peptidylarginine deiminase. This resulted in loss of calcineurin phosphatase activity and decreased calmodulin activation of calcineurin due to an apparent reduced affinity of calmodulin for deiminated bovine brain calcineurin. Through
amino acid analysis of calcineurin which was protected from loss of activity upon deimination by addition of calmodulin, we found that one or possibly two arginines relevant to these observed effects were being modified by the deiminase. We conclude that at least one arginine in calcineurin is important for calmodulin binding and is likely located at the calmodulin binding site of the CaN A subunit.

Because of this conclusion, further localization of this arginine residue(s) was necessary. To this end, a truncated form of rat brain calcineurin was expressed in Sf21 cells in which all the arginines in the C-terminus of CaN A, except for those in the previously defined calmodulin domain, were deleted. Interestingly, deimination of this truncated form of calcineurin resulted in the incorporation of 0.5 mol/mol less citrulline than the full-length expressed calcineurin and no loss of phosphatase activity. The reduced amount of citrulline in truncated calcineurin could be due to truncation of an arginine which is normally deiminated in full-length calcineurin. Therefore, deimination of an arginine not present in the truncated calcineurin may be responsible for the loss of activity and reduced calmodulin binding of the full-length rat and bovine brain calcineurins.

Since, in the 14 kDa C-terminus of rat brain CaN A, no other arginines exist outside of the previously defined calmodulin binding or autoinhibitory domains, the decreased amount of citrulline in the truncated calcineurin might mean
that the additional arginine being modified in the full-length calcineurin is in its autoinhibitory region. If this is the case, then one might hypothesize that calcineurin may contain two calmodulin binding domains, the calmodulin binding domain as previously described by Kincaid and Martin (17) and the sequence corresponding to or overlapping the autoinhibitory domain as described by Hashimoto et al (18). There is precedence for calmodulin binding to two distinct sites on an enzyme; in the phosphorylase kinase catalytic subunit, the C-terminal region contains two distinct calmodulin sites which calmodulin binds simultaneously (204). The autoinhibitory domain even contains a sequence motif in which every fourth residue in the sequence is a basic residue. This is reminiscent of the basic amphiphilic α-helix motif to which calmodulin binds (59), although the calcineurin autoinhibitory domain is not a true amphiphilic helix. Perhaps calmodulin could bind to the second putative site on calcineurin because of the similarity of the autoinhibitory domain to the amphiphilic helix. It well known that specific sequences to which calmodulin will bind are not well defined, but show considerable sequence diversity (59).

Another hypothesis which may be made concerning the activation of calcineurin by calmodulin is that the calmodulin binding and autoinhibitory domains of calcineurin may not be separated by 50-60 residues like previously thought (17,18,61), but may actually be overlapping. Perrino et al (205) and studies in this dissertation have shown that truncating CaN A so
that the previously defined autoinhibitory domain is deleted
results in an enzyme which can be further activated by addition
of calmodulin. This indicates that sequences N-terminal to the
identified autoinhibitory domain also contain autoinhibitory
properties. Therefore, the actual autoinhibitory domain of
calcineurin may extend and be adjacent to the previously defined
calmodulin binding domain of calcineurin. Because of this and
the idea that calmodulin may bind not only to the previously
described calmodulin binding domain of calcineurin but also its
autoinhibitory domain, we speculate that the domain organization
of the calmodulin binding and autoinhibitory regions of
calcineurin may not be so different from that of other
calmodulin-dependent enzymes. The calmodulin binding and
autoinhibitory domains of calcineurin may overlap, as do those
of smooth muscle myosin light chain kinase (smMLCK), CaM-kinase
II, and the catalytic subunit of phosphorylase kinase
(62,63,207).

The second phosphatase described in this dissertation is
PTP-1, a rat homolog of PTP-1B, a protein tyrosine phosphatase
thought to be involved in the attenuation of the insulin signal
(172,174,175). The activity of PTP-1 was found to be regulated
by compounds derived from cinnamon, compounds which potentiate
insulin's activity in the rat epididymal fat cell assay. One
fraction derived from cinnamon, the void fraction, is an
irreversible inactivator of PTP-1. Another fraction, the yellow
fraction also inhibits PTP-1, but does not irreversibly
inactivate PTP-1. Therefore, the cinnamon compounds may be acting to increase insulin signalling by inhibiting a PTPase which normally dephosphorylates the insulin receptor or a subsequent protein in the insulin signalling pathway, resulting in down-regulation of the receptor signal.

However, the cinnamon compounds not only act to inhibit PTP-1, but they also act to increase insulin receptor kinase catalytic domain autophosphorylation directly. Therefore, the cinnamon compounds may have a dual function in insulin signalling, causing stimulation of insulin receptor kinase autophosphorylation while inhibiting PTPases which would attenuate the kinase signal. Wortmannin decreases the biological potentiation of insulin by inhibiting PI 3'-kinase activity (114,115). In these studies, wortmannin was found to decrease the biological response to insulin and cinnamon similarly. This indicates that the cinnamon compounds are affecting element(s) in insulin signalling upstream of PI 3'-kinase. Since insulin receptor kinase autophosphorylation is activated by the cinnamon compounds in vitro, the insulin receptor itself is a good candidate for an in vivo target for the cinnamon compounds.

If the cinnamon compounds do have a dual role in insulin signalling, then the above wortmannin result implies that the relevant PTPase(s) would also be acting upstream of PI 3'-kinase. The obvious possible substrates for this PTPase which are upstream of PI 3'-kinase are IRS-1 and the insulin receptor
kinase itself. Although IRS-1 is known to be dephosphorylated in vivo (146,147), nothing is known about the identity of the PTPase(s) involved. More is known about the dephosphorylation of the insulin receptor kinase. A strong candidate PTPase for the dephosphorylation of the receptor kinase and attenuation of the kinase signal in vivo is PTP-1B (172,174,175), which is 97% identical to the rat PTP-1 used in these studies. Perhaps one of the targets of the cinnamon compounds in the rat fat cell assay is PTP-1B.

Studies now need to be undertaken to determine if these in vitro results suggesting that certain cinnamon compounds activate the insulin receptor kinase and inhibit a PTPase hold for in vivo systems. This may include the determination of the phosphorylation state of the receptor kinase and PTPase activities in adipocytes in response to these cinnamon compounds. This and further characterization of the identity of the cinnamon compounds responsible may lead to identifying another possible treatment for insulin resistance.
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This dissertation is dedicated to my parents, Jane and Albert Imparl, who always taught me that I could aspire to be anything that I wanted to be.