

Studies on Bovine Eye Retinal Calcineurin

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By

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ABSTRACT

Calcineurin (CaN), a member of ser/thr protein phosphatase, was cloned from bovine retina. The peptide sequence of CaN A subunit is consisted of 511 amino acid residues. A 10 amino acid (A-T-V-E-A-I-E-A-D-E-A) deletion before the autoinhibitory domain was observed in bovine retina CaN A compared to bovine brain CaN A. The study on CaN activity and regulation demonstrated that different metal ions have different effects on its phosphatase activity. Ni^{2+} was found to be the strongest stimulator while Zn^{2+} was found to inhibit CaN phosphatase activity. Mn^{2+} was a relatively less effective stimulator compared to Ni^{2+} . Fe^{2+} was also able to stimulate CaN phosphatase activity; in contrast, a previous study found Fe^{2+} slightly inhibited bovine brain CaN activity. The residues at 97-201 were found to be essential for bovine retina CaN A phosphatase activity. The residues at 407-456 also had an inhibitory effect on CaN A phosphatase activity in addition to the previously known auto inhibitory domain at 457-480. These observations suggest that bovine retina CaN A might possess some distinct structural characteristics compared to bovine brain CaN A.

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DEDICATION

This Work is Dedicated

To

My Mom, Dad and Aunt

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LIST OF ABBREVIATIONS

BAD	Bcl-2 Associated Death promoter protein
Bcl-2	B cell lymphoma 2
Bcl-x2	Basal cell lymphoma-extra 2
AtCBL	<i>Arabidopsis thaliana</i> Calcineurin B-like protein
CaM	Calmodulin
cAMP	3',5', Cyclic Adenosine Monophosphate
CaMBP ₈₀	Calmodulin Binding Protein 80 kDa
CaN	Calcineurin
CaN A	Calcineurin α Subunit
CaN B	Calcineurin β Subunit
cAMP-PK	cAMP-dependent Protein Kinase
CHP	Calcineurin Homologous Protein
CIB	Ca ²⁺ - and Intergrin-Binding Protein
CsA	Cyclosporin A
CaMPDE	Calmodulin-stimulated Cyclic Nucleotide Phosphodiesterase
C/EBP	CCAAT Enhancer Binding Protein
Cytc	Cytochrome c
DARPP-32	Dopamine- and cyclic AMP-Regulated Phosphoprotein with molecular weight 32 kDa
FK506	Tacrolimus
FasL	Fas Ligand
GATA2	GATA binding protein 2
GLP-1	Glucagon-Like Peptide-1

Glut2	Glucose Transporter
IGF-1	Insulin-like Growth Factor-1
IL-2	Interleukin-2
IOP	Intraocular Pressure
MEF2	Myocyte Enhancing Factor 2
NCS-1	Neuronal Calcium Sensor
VSMC	Vascular Smooth Muscle Cell
Ca ²⁺	Calcium
K ⁺	Potassium
<i>E. coli</i>	<i>Escherichia coli</i>
DNA	Deoxyribonucleic Acid
cDNA	Complementary Deoxyribonucleic Acid
mRNA	Messenger Ribonucleic Acid
<i>m</i> -calpain	Calpain that activated by milli-molar concentration of Ca ²⁺
G proteins	Guanine Nucleotide-binding Proteins
GPCR	G Protein Coupled Receptor
PLCβ	Phospholipase Cβ
DAG	Diacylglycerol
PKC	Protein Kinase C
Ins(1,4,5)P ₃	Inositol-1,4,5-triphosphate
MLP	Muscle LIM Protein
JNK	c-Jun N-terminal Kinase
MAPK	Mitogen-Activated Protein Kinase
ON	Optic Nerve
pBAD	Phosphorylated BAD

LB	Luria Broth
RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction
EGTA	Ethylene Glycol Tetraacetic Acid
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
RGC	Retinal ganglion cell
pNPP	para-Nitrophenylphosphate
PCR	Polymerase Chain Reaction
Sm-MHC	Smooth Muscle Heavy Chain
PP3	Protein Phosphatase 3

LIST OF AMINO ACIDS

A Alanine

R Arginine

N Asparagine

D Aspartic Acid

C Cysteine

E Glutamic Acid

Q Glutamine

G Glycine

H Histidine

I Isoleucine

L Leucine

K Lysine

M Methionine

F Phenylalanine

P Proline

S Serine

T Threonine

W Tryptophan

Y Tyrosine

V Valine

1.0 REVIEW OF THE LITERATURE

Cells, in order to maintain their growth, development, defense as well as homeostasis, have the ability to perceive and respond to their micro-environment. Cell signaling is a part of complex communication system that governs and coordinates various cellular activities. In addition, signal transduction refers to any process by which a cell converts one type of signal or stimulus into another. Most signal transduction processes involve ordered sequences of biochemical reactions inside the cell, which are carried out by proteins/enzymes and activated second messengers. Second messengers including calcium (Ca^{2+}) and cyclic adenylyl monophosphate (cAMP) play a major role in intracellular signal transduction. Usually, cytosolic Ca^{2+} concentration is maintained at a low level by sequestration in the smooth endoplasmic reticulum and mitochondria. Release of Ca^{2+} from endoplasmic reticulum and mitochondria results in the elevation of cytosolic Ca^{2+} level accompanied with the binding of Ca^{2+} to certain signaling proteins, which then become activated (1).

Among all the Ca^{2+} binding proteins, calmodulin (CaM) is one of the most extensively studied proteins (2). It was discovered as a protein activator of cyclic nucleotide phosphodiesterase (PDE) by Cheung (3, 4). Later, Kakiuchi and Yamazaki demonstrated the existence of Ca^{2+} -stimulated PDE in rat brain (5). In addition, they discovered an endogenous protein factor in brain that could enhance the Ca^{2+} sensitivity of the enzyme (5). It was established subsequently that the protein factor was identical to the protein activator of PDE later called CaM, which was originally discovered by Cheung (3, 4). The stimulation of PDE

required the presence of both Ca^{2+} and CaM (8).

CaM is widely expressed in all cell types (6, 7). CaM is a heat-stable small acidic protein having a molecular mass of 17 kDa (8). It contains four “EF-hand” motifs which have high affinity toward Ca^{2+} and bind to four Ca^{2+} molecules (9). Previous studies have suggested a mechanism of action of CaM in the activation of various enzymes (Figure 1) (3, 10-12). CaM exhibits high affinity Ca^{2+} -binding capacity. In the presence of Ca^{2+} , CaM undergoes a conformational change, which enables it to bind to a number of target proteins for specific cellular responses (13, 14), hence, it mediating multiple regulatory activities (10). CaM is involved in numerous biological pathways including apoptosis, Ca^{2+} metabolism, cyclic nucleotide metabolism and muscle contraction (15). In fact, CaM acts as a Ca^{2+} sensor and signal transducer (Figure 2) (16). Calcineurin (CaN) is one of the CaM-binding proteins that can directly bind to Ca^{2+} .

1.1 Discovery of CaN

CaN was originally discovered as a contaminant in preparation of CaM-stimulated cyclic nucleotide phosphodiesterase (CaMPDE) which inhibited PDE activity (11, 17-19). It was purified from bovine brain based on its ability to undergo a Ca^{2+} -dependent association with CaM (19-22). It was designated as CaM binding protein 80 (CaMBP₈₀) because it binds to CaM and has a molecular mass approximately 80 kDa (21). It contained two distinct subunits, CaN A and CaN B, with molecular mass of 60 kDa and 20 kDa, respectively. CaMBP₈₀ was demonstrated to have inhibitory activity on CaMPDE by competing for binding to CaM with the latter (10, 17). Subsequently, CaMBP₈₀ was re-named as CaN due to

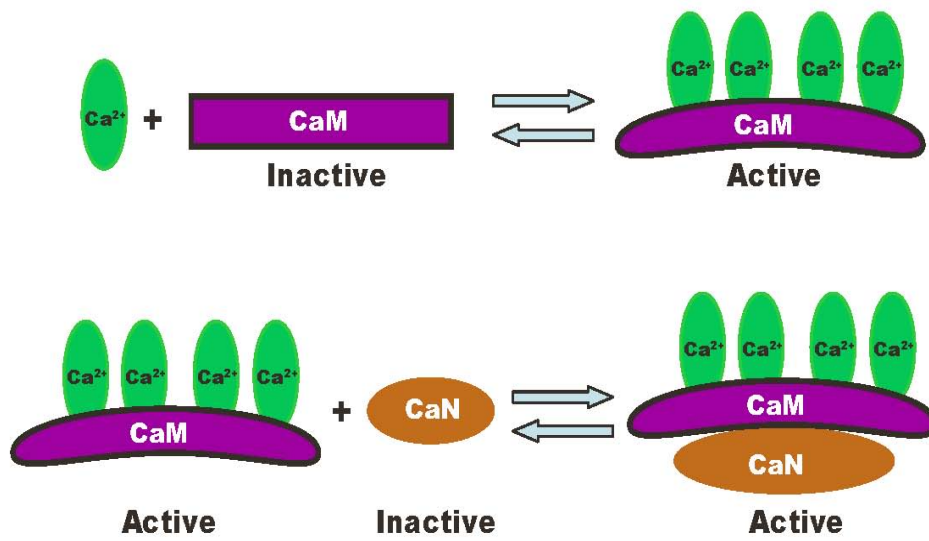


Figure 1. Schematic diagram of mechanism of CaM activation and action on its target protein (i.e. CaN).

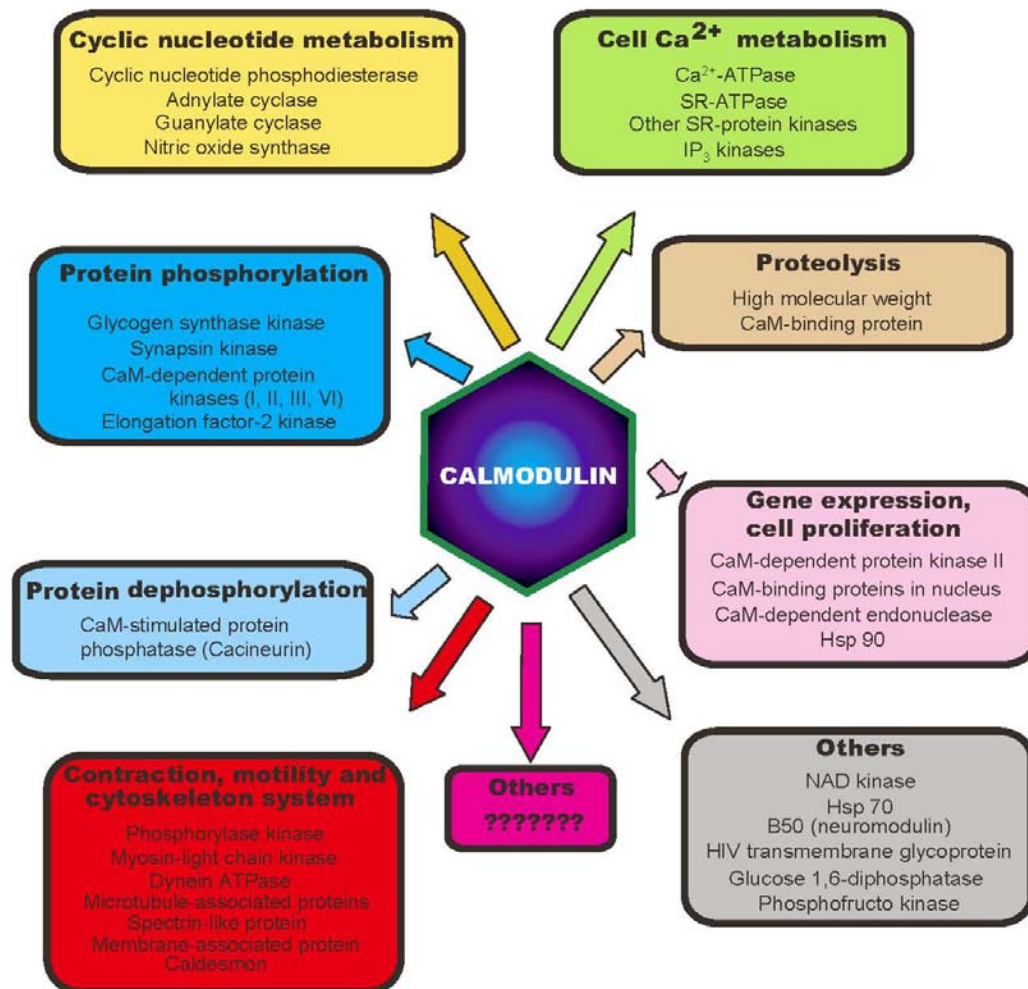


Figure 2. Various proteins and enzymes that are regulated by CaM.

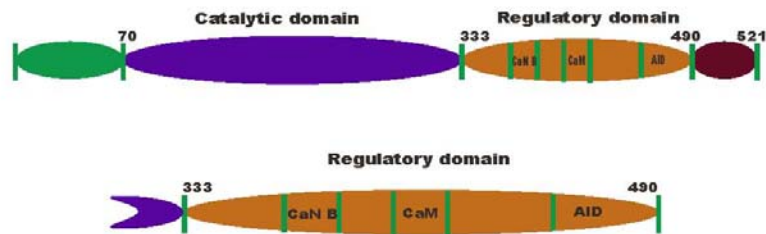
presence of high amount in brain and intrinsic Ca^{2+} -binding property (23). Later, Stewart *et al.* established that CaN contained CaM-dependent protein phosphatase activity (24).

Moreover, the serine/threonine protein phosphatase family is consisted of protein phosphatase 1 (PP1), PP2A, PP2B (AKA PP3) and PP2C. Now, it is clear that CaN is a CaM-dependent serine/threonine protein phosphatase 3 (PP3). Independently, studies on protein phosphatase-2B (PP2B) revealed that CaN and PP2B are identical proteins (24). Except PP2C, all other members share the same sequence homology in their catalytic domain and are evolutionarily related (25-27). The original classification of PP2C along side PP1, PP2A and CaN does not hold at the primary sequence level. PP2C is considered to be in a separate superfamily (28). Furthermore, all the mammalian serine/threonine protein phosphatases are separated into two classes (26, 27, 29, 30). Class-1 protein phosphatases were found to dephosphorylate the β -subunit of phosphorylase kinase while class-2 protein phosphatases dephosphorylate the α -subunit of phosphorylase kinase.

1.2 CaN Properties

CaN is a heterodimer containing CaN A and CaN B subunits (Figure 3). Mammalian CaN A exists in α , β , and γ isoforms while CaN B exists in B1 and B2 isoforms. The first suggestion that CaN has tissue-specific isoforms was supported by several independent studies (31). On one hand, CaN was only found to have a very high concentration in neuronal cells by radioimmuno-assay using an antibody raised against purified bovine brain CaN (32). On the other hand, CaN activity was found to be ubiquitously expressed in mammalian tissues (33, 34). Various investigators purified CaM-dependent phosphatases showing similar

CaN-A



CaN-B

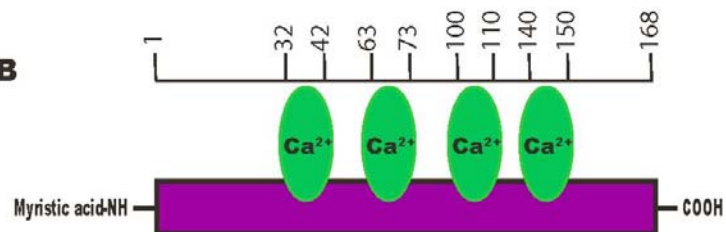


Figure 3. Schematic representation of domain organizations of CaN A and CaN B subunits. CaN A contains four domains include: catalytic domain, CaN B-binding domain, CaM-binding domain and auto-inhibitory domain (AID). CaN B contains four EF-hand Ca^{2+} -binding motifs.

structure as the bovine brain CaN from different mammalian tissues including platelet (35), skeletal muscle (33, 36), cardiac muscle (36), placenta (37) and pancreas (38, 39). In mammals, CaN A has a molecular mass range between 57-59 kDa depending on isoform. The size of CaN A can be approximately 20% larger in lower eukaryotes (40-46).

Nevertheless, all CaN A genes encode for a catalytic domain homologous to other serine/threonine protein phosphatases and three regulatory domains that distinguish CaN from others. These three domains include the CaM-binding domain, CaN B-binding domain and auto-inhibitory domain (Figure 3). These domains have been identified through biochemical mapping procedures. The X-ray structures of CaN confirm the identification of residues involved in these regulatory domains and indicate the auto-inhibitory domain forms a α -helix that binds to the substrate-binding site of the catalytic domain (47). The NH₂ and COOH termini are highly variable among species and among CaN A genes within the same organism (48-50). The function of the variability is unknown, but they might play a role in substrate-specificity and localization.

CaN B has a molecular mass of 18 kDa and contains four EF-hand Ca²⁺ binding sites (Figure 3). The amino acids sequence of CaN B is also highly conserved among species. For example, mammalian CaN B is sharing 86% amino acid sequence homology with insect CaN B. There are two mammalian CaN B subunits. One is ubiquitously expressed along with CaN A α and CaN A β while the other one is only found in testes along with CaN A γ (51-53). The mature CaN B subunit has a myristoylation site at its NH₂-terminus region (54).

However, several studies have found that the myristoylation is required neither for membrane association nor for enzymatic activities (55, 56). Although the myristoylated CaN

B had substantial thermal stability relative to the non-myristoylated protein (56), the physiological role of CaN B myristoylation is unknown. Furthermore, the primary sequence determination of CaN B demonstrated homology with CaM and troponin C (57). EF-hand proteins have been classified into 39 distinct subfamilies including from two to eight EF-hand domains (58). Sequence alignments revealed that CaN B proteins belong to one subfamily of EF-hand proteins (59). CaN B has shown to be homologous to many EF-hand containing Ca^{2+} -binding proteins including Ca^{2+} - and integrin-binding protein (CIB), a neuronal Ca^{2+} sensor (NCS-1), a protein p22/CHP (CaN homologous protein) *etc.* (60-62). Some studies have showed an overlapping function between CaN B and its homologous proteins (62-64).

1.3 Physiological Roles of CaN

1.3.1 In mammals

1.3.1.1 cAMP metabolism and microtubule assembly

In contrast to many other Ca^{2+} -binding proteins such as troponin C, parvalbumin, and S-100 which have a limited distribution in certain tissues and restricted physiological functions; CaN is ubiquitously distributed in all tissues and possesses multifunctional phosphatase activity (65, 66). Several studies have proposed that CaN might play a role in cAMP metabolism (67). It has been demonstrated *in vitro* that CaN dephosphorylates CaMPDE, which exists in different isozymic forms (68-72). CaMPDE is involved in the regulation of cellular cAMP level by catalyzing the conversion from cAMP to AMP. Furthermore, CaMPDE can be phosphorylated; and the phosphorylation results in decrease in affinity towards CaM, which in turn causes a decrease in PDE activity (73, 74). However, CaN is able to dephosphorylate the phosphorylated CaMPDE and bring back its binding

affinity towards CaM accompanied with recovered PDE activity (73, 74). These findings fully supported that CaN is involved in cAMP metabolism.

In addition, CaN can modulate cAMP effect by dephosphorylation (22, 75, 76). When cellular cAMP level is elevated, cAMP-dependent protein kinase (cAMP-PK) will be activated. cAMP-PK target proteins including Inhibitor-I and DARPP-32 will be phosphorylated. These phosphorylated proteins have an inhibitory effect on protein phosphatase-1, which can dephosphorylate Inhibitor-I and DARPP-32. Thus, the cAMP signaling will be amplified until another phosphatase, CaN, becomes activated; hence, CaN will dephosphorylate Inhibitor-1 and DARPP-32. In this case, cAMP signaling will be terminated. And the inhibition on protein phosphatase-1 will be removed.

In addition, microtubule-associated protein-2 and tau factor are involved in microtubule assembly. Both proteins can be phosphorylated by either cAMP-PK or CaM/Ca²⁺-dependent protein kinase (77). The phosphorylation will inhibit the microtubule assembly. However, it has been found that both phosphorylated proteins can be dephosphorylated by CaN. The inhibition can be aborted by dephosphorylation. Thus, it is possible that CaN is involved in regulating microtubule assembly.

1.3.1.2 CaN and skeletal muscle metabolism

Moreover, CaN has been revealed to transduce Ca²⁺ signals from motor neurons to myofibers (78-80). Signals evoked by motor neurons can induce changes in metabolic gene expression in skeletal muscle (81). Skeletal muscle is generally divided into two types partially dependent on the metabolic properties: fast glycolytic and slow oxidative fibers. The former utilizes energy derived from glycolysis while the latter utilizes energy mainly

derived from lipid oxidation also known as the Krebs cycle. The impact of CaN activation on skeletal muscle metabolic properties including glycogenesis, glycolysis and lipid oxidation were studied on transgenic mice that over-express activated CaN (82). As a result, the level of glycogenesis was increased while the level of glycolysis was decreased comparing to wild type mice. In contrast, the level of lipid oxidation was increased.

Additionally, the mRNA levels of genes encoding for proteins involved in glycogenesis such as hexokinase (*HK2*) and pyruvate dehydrogenase kinase 4 (*Pdk4*) were up-regulated while genes encoding for proteins involved in glycolysis such as aldolase (*Aldoa*), glyceraldehydes-3-phosphate dehydrogenase (*Gapdh*) and muscle 6-phosphofructokinase (*Pfkm*) were down-regulated. In contrast, mRNA levels of genes for lipid metabolism including lipoprotein lipase (*Lpl*), fatty acid transporter (*cd36*), carnitine palmitoyl-CoA transferase 1 (*Cpt 1*), mitochondrial carnitine/acylcarnitine translocase (*Slc25a20*), 2,4-dienoyl-CoA reductase 1 (*Decr 1*) and very long chain acyl-CoA dehydrogenase (*Acadvl*) were found to be significantly elevated in MCK-can mice compared to the wild type. The alterations in gene expression pattern were associated with decreased glucose utilization and increased glycogen storage. Thus, activated CaN can regulate energy substrate utilization in skeletal muscle by up-/down-regulating gene expression of proteins involved in glucose or lipid metabolism.

1.3.1.3 CaN and apoptosis

CaN has been demonstrated to be involved in cell apoptosis (83) and the regulation of ion channels (84). Ca^{2+} signalling is upstream of some pathways that lead to apoptosis (85), for example, neuronal cell death through glutamate-induced excitotoxicity and cell death in T

cells (86, 87). Although many studies demonstrated the link between Ca^{2+} influx and cell death, the immediate downstream events were not revealed. Later, a Ca^{2+} signal mediator, CaN, was chosen to be a potential inducer in apoptosis (88). Apoptosis is a mechanism to remove cells, which experience inappropriate signals (89-91). For example, stimulation of T cell receptor on immature T cells drives them into apoptosis rather than proliferation (92, 93). In the study, CaN was demonstrated to be an inducer of Ca^{2+} -triggered apoptosis in the absence of growth factor in mammalian cells (88).

Thereafter, a mechanism of CaN -inducing apoptosis was proposed (94). L-glutamate triggers Ca^{2+} influx, which in turn activates CaN. CaN dephosphorylates a pro-apoptotic factor BAD, which is a member of the Bcl-2 family. The dephosphorylated BAD translocates from cytosol into mitochondria where heterodimerization of BAD and Bcl-X2 takes place. Then, apoptosis is initiated. Furthermore, the CaN -induced BAD translocation and apoptosis were found to be inhibited by CaN inhibitors. In addition, certain pathological states including prostate cancer and neuronal cell death were found to be linked to CaN -induced apoptosis (95-97).

1.3.1.4 CaN and ischemia

Myocardial ischemia is a condition in coronary artery disease. Heart tissue will be damaged due to lack of oxygen and other nutrients result from the blockage of artery. This form of heart failure is thought to be caused by loss of cardiac myocytes through apoptosis (98-100). CaN has been revealed as an essential regulator of cardiomyocyte apoptosis (101). CaN has been found to be involved in regulation of both pro- and anti-apoptotic factors (102-104).

Our laboratory has investigated the alterations of CaN activity and expression in ischemic myocardium and demonstrated that a significant increase in CaN activity was observed in both animal model and patient tissue (105). However, the expression of intact CaN A was lower in ischemic tissue compared to normal tissue (105). Interestingly, a proteolysed form of CaN A, from 60 kDa to 46 kDa, was observed in ischemic tissue while it was not observed in normal tissue. Furthermore, the 46 kDa CaN A was found to be more active than that of the intact form (105). Additionally, the expression of *m*-calpain, a major Ca^{2+} signal mediator, was found to be increased in ischemic tissue. It was also demonstrated that the truncated form of CaN A was due to the controlled cleavage of CaN A by *m*-calpain in vitro (105). Thus, during myocardial ischemic condition, the elevated influx of Ca^{2+} causes activation of *m*-calpain, which in turn activates CaN through limited cleavage. The activated form of CaN will further activate pro-apoptotic factors to initiate programmed cell death in myocardium.

1.3.1.5 CaN and epilepsy

Epilepsy is a condition of seizure disorder caused by neuron damage in brain. CaN has attracted attention as a factor involved in this pathological condition due to its high expression level in brain and involvement in apoptosis pathways. Our laboratory has focused on the expression of CaN and its interaction with various proteins in epileptic tissue from animal model (106). The expression and activity of CaN were found to be increased in epileptic tissue compared to normal tissue (106). Moreover, the interaction between CaN and *m*-calpain was stronger in epileptic tissue than that of normal tissue (106). Since *m*-calpain can partially degrade CaN A subunit, it is highly possible that the elevated CaN activity was

partially contributed by *m*-calpain cleavage (106). Furthermore, immunoprecipitation showed stronger interaction between CaN and certain apoptotic factors including Bcl-2, caspase-3 and p53 (106). All these data together suggest that CaN might play an important role in initiating apoptosis pathways in neuronal cells (107).

1.3.1.6 CaN cleavage

Lakshmikuttyamma *et al* demonstrated the proteolysis of CaN by *m*-calpain *in vitro* (108). A 46 kDa fragment of CaN A was observed when it was incubated with *m*-calpain in the presence of Ca^{2+} . Two fragments, 54 and 48 kDa, were observed in the presence of CaM and Ca^{2+} . No proteolysis of CaN B was observed in either condition (Figure 4) (108). CaN phosphatase activity was measured to see whether the activity was altered by the degradation. The experiment showed a 50% increase in activity upon degradation either in the presence of Ca^{2+} or Ca^{2+} /CaM. The study also investigated whether the proteolysed fragment of CaN A was CaM-independent or not. The 46 kDa and 48 kDa fragments did not contain CaM-binding site while the 54 kDa fragment did (Figure 4) (108). The study concluded that the proteolysis of CaN by *m*-calpain increased its activity and converted it into a CaM-independent form. It is highly possible that activated *m*-calpain and CaN activity modification could contribute to the bridge between cell survival and intracellular Ca^{2+} concentration regulation.

1.3.1.7 CaN-NFAT in skeletal muscle

Studies based on immune system have revealed that CaN plays a crucial role in T-cell activation (109-115). Activated CaN dephosphorylates the nuclear factor of activated T-cells (NFAT), which translocates into the nucleus. NFAT will up-regulate transcription of T-cell

gene encoding interleukin (IL-2) and lead to T-cell activation (114, 115). The NFAT proteins represent a family of transcription factors widely expressed in mammalian tissues. There are at least four members of NFAT including NFATc1, NFATc2, NFATc3 and NFATc4.

Recent studies have demonstrated that the role of NFAT signaling is by no means restricted to the immune system. Physiological and developmental signals in cells such as myocytes and neurons stimulate intracellular Ca^{2+} transients and lead to activation of protein phosphatase, which in turn dephosphorylates the cytoplasmic subunit of NFAT transcription complexes (NFATc). In fact, NFATs are Ca^{2+} -signaling mediators in many mammalian tissues including skeletal muscle, smooth muscle, cardiac muscle and pancreas. CaN-NFAT signaling, in cooperation with many other signaling factors, can regulate a number of cellular events (116-118).

For instance, insulin-like growth factor I (IGF-I) plays a major role in muscle differentiation, induction of myocyte hypertrophy and regeneration of skeletal muscle (119-121). Loss of IGF-I signaling during muscle development might result in defective growth of skeletal muscle (122, 123). Various studies demonstrated the cross-talk between CaN-NFAT and IGF-I signaling. For example, the embryonic IGF-I expression in differentiating somites and branchial arches is largely dependent on NFATc3 (124), which is the first NFAT protein to be translocated into the nucleus during skeletal myoblasts differentiation (125). The clustered NFAT consensus binding sequences were found in two highly conserved regions that identified in the 5'-flanking regions of the IGF-I exon 1 promoter (126).

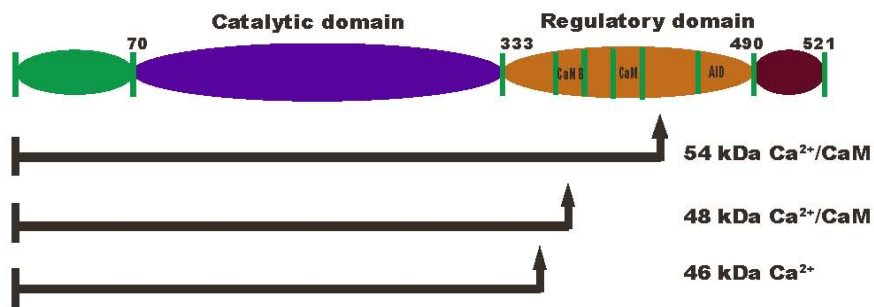


Figure 4. *m*-Calpain cleavage sites on brain CaN. Schematic diagram represents the domain organization of CaN A. Proposed locations of the *m*-calpain cleavage sites within CaN A giving rise to the indicated proteolytic fragments, as a result of cleavage in the presence of Ca^{2+} or $\text{Ca}^{2+}/\text{CaM}$ as indicated. CaN B, CaN B binding domain; CaM, CaM binding domain; AID, auto inhibitory domain.

In addition to these NFAT binding sites, some known myogenesis regulators such as CEBP, E-box and MEF2 binding sites were also found near the IGF-I exon1 promoter region. This implies a possible pathway to regulate IGF-I gene expression during muscle development. Indeed, these NFAT binding sequences were found to be responsive to CaN-NFATc3 signaling; and IGF-I gene expression was induced by NFATc3 in the presence of activated CaN. Thus, the identification of CaN-NFAT responsive sequence in the IGF-I gene implies a possible mechanism to regulate muscle development and homeostasis by the two pathways.

For instance, IGF-I and CaN-NFAT signalling were revealed to be responsible for skeletal muscle hypertrophy (127-129). The hypertrophic response was observed in both transgenic mice treated with IGF-I and transgenic mice expressing constitutively activated CaN (127). Essentially, IGF-I can activate CaN by increasing intracellular Ca^{2+} concentration. Once CaN is activated, NFAT is dephosphorylated and translocated into nucleus, where the expression of GATA-2, a skeletal muscle hypertrophy marker, is induced. GATA-2 will further activate gene expression programs toward development of hypertrophic responses. On the other hand, the hypertrophy was repressed in transgenic mice expressing a dominant-negative form of CaN or by addition of CaN inhibitors.

1.3.1.8 CaN signaling in smooth muscle

Vascular smooth muscle cells (VSMCs) can undergo phenotypic modulation and act as a major component of thickened arterial intima (130). Changes in the expression pattern of smooth muscle-myosin heavy chain (Sm-MHC) are accompanied with the development of phenotypic modulation (131-134). The Sm-MHC gene is a downstream target of GATA-6

which plays a role in the VSMC differentiation (135). GATA-6 is a zinc-finger protein belongs to a family of transcription regulators.

It has been revealed the connection between CaN-NFAT and GATA-6 during smooth muscle differentiation (130). First of all, Sm-MHC promoter was not activated when GATA-6 could not bind to the gene. CaN was also required for the transcription activation of Sm-MHC gene because the the promoter activity was largely reduced when VSMCs were treated with CaN inhibitors during differentiation (130). Moreover, the binding of GATA-6 to Sm-MHC gene was significantly inhibited in the presence of CaN inhibitors. In other words, CaN is required for GATA-6 binding to the DNA. The study also showed the interaction between GATA-6 and NFATc1 which is the major isoform of NFAT found in smooth muscle, through the zinc-finger domain of GATA-6. In addition, Sm-MHC gene expression level was reduced with the treatment of CaN inhibitors in differentiated VSMCs (130). Thus, CaN-NFAT, in cooperation with GATA-6, are involved in regulation Sm-MHC gene expression. These three proteins together build up a possible signaling pathway for the maintenance of differentiated phenotype in VSMCs. This finding may lead to designing therapeutic agents against diseases involving vascular injury.

1.3.1.9 CaN-NFAT and cardiac hypertrophy

Cardiac hypertrophy is a major risk factor for the development of heart failure. In response to various pathologic stimuli, the myocardium undergoes growth in length and/or width in order to increase cardiac pump function and decrease ventricular wall tension (136, 137). The initial stimuli can be divided into two categories: biomechanical and stretch-sensitive mechanisms, and neurohumoral mechanisms (138). A number of ligands such as

angiotensin II, catecholamines, neuregulin and IGF-I *etc.* are sensed by cardiac myocytes through various membrane-bound G-protein-coupled receptors (GPCRs) (138). These receptors will initiate a number of intracellular signaling pathways to mediate cardiac growth response (138). Alterations of gene expression in the nucleus and concentrations of proteins in the cytoplasm will be involved in such signaling pathways to coordinate hypertrophic response (138).

CaN was identified to play a central role in hypertrophic responses (139), such as ligands/G-protein-coupled receptors (GPCRs) induced and biomechanically induced cardiac hypertrophy (138). Several studies have provided evidences to demonstrate the importance of G-protein-coupled receptors in pathological cardiac hypertrophy (140-143). For example, overexpression of $G_{\alpha q/\alpha 11}$, a G protein, increased the tendency of cardiac hypertrophy under pressure-overload condition and resulted in heart failure (140-143). However, inhibition of $G_{\alpha q/\alpha 11}$ resulted in the decrease of such hypertrophic response under pressure-overload condition (144, 145). In addition, $G_{\alpha q/\alpha 11}$ induced hypertrophy is not required for normal heart functional compensation (138). Furthermore, CaN-NFAT signaling was proposed to be part of the downstream events of GPCR-induced hypertrophy (138). G proteins of $G_{\alpha q/\alpha 11}$ are activated through binding of ligands such as Ang II, Endo-1 and catecholamines. Activated G proteins are coupled to phospholipase C β (PLC β). This coupling will induce the generation of diacylglycerol (DAG), which will activate protein kinase C (PKC). Activation of PKC will lead to production of inositol-1,4,5-triphosphate (Ins(1,4,5)P $_3$) (146). Accumulation of Ins(1,4,5)P $_3$ results in release of Ca $^{2+}$ from endoplasmic reticulum or the nuclear envelope. Increase of intracellular Ca $^{2+}$ concentration will lead to activation of various proteins such as

CaM, CaMPK and CaN. These proteins will further activate prohypertrophic gene expression by dephosphorylating and translocating NFAT (147, 148).

Addition to GPCR initiated mechanism, biomechanically induced hypertrophy also involves CaN-NFAT signalling pathway (149, 150). There are two sensing apparatus that have been proposed to transduce biomechanical stress signals through attached signalling molecules (138). One is consisted of three components including extracellular matrix, integrins, which are heterodimeric transmembrane receptors, and intracellular cytoskeleton (151). The other sensing apparatus is thought to be located at the level of Z-disc within each sarcomere (152). The small LIM-domain protein, muscle LIM protein (MLP), is anchored to specific proteins at the Z-disc. It has been proposed to act as an internal stretch sensor through a complex of transducing proteins including CaN-NFAT signalling pathway (149, 150, 152).

In both mechanisms, activation of CaN and translocation of NFAT into the nucleus are proposed to be the crucial events which lead to cardiac hypertrophy. For example, inhibition of CaN resulted in reduced hypertrophy response upon pressure-overload condition (153). Transgenic mice with disrupted expression of NFATc3 were demonstrated to have reduced myocardial growth in response to activated CaN gene or in pressure-overload condition or other extracellular signals (147). Additionally, cardiac hypertrophy induced by CaN-NFAT signalling can be negatively regulated by kinases such as p38 and c-Jun N-terminal kinases (JNKs), which are activated through mitogen-activated protein kinase (MAPK) (154-156). These kinases are proposed to antagonistically attenuate the dephosphorylation of NFAT by CaN.

1.3.1.10 CaN and glaucoma

Glaucoma is one of the leading causes of irreversible blindness in the United States (157). The principle factor for developing glaucoma is the elevated intraocular pressure (IOP), which further leads to retinal ganglion cell (RGC) death (158, 159). The RGC death is driven by apoptosis pathways (160-163). However, the upstream mechanisms initiating the apoptosis were not known (164). The primary treatment is to lower the IOP; however, many patients still lose vision despite aggressive treatment (160). In order to improve the efficiency of treatment, the mechanism of how elevated IOP resulted in RGC death needed to be investigated.

CaN has been found to be a critical regulator of Ca^{2+} -induced apoptosis pathways (164). It was revealed to dephosphorylate the proapoptotic Bcl-2 family member, BAD (165). The dephosphorylated BAD further leads to cytochrome *c* (cyt *c*) release, caspase activation, and apoptotic cell death (165, 166). Recently, one research group has demonstrated a possible pathway, which mediates the effect of increased IOP to RGC death (164). The major finding from this study is the correlation between elevated IOP and cleavage of CaN A. Western blot analysis was done by using antibody specific for full length (60 kDa) CaN A and truncated (46 kDa) CaN A (167, 168). The truncated CaN A was observed in half of the eyes with elevated IOP for 5 days and in all eyes with elevated IOP for 10 days. The observation from an experiment based on the DBA/2J mouse model of spontaneous glaucoma further confirmed the result. In addition, experiments were performed to verify the limited cleavage of CaN A was a specific apoptosis event.

According to Berkelaar *et al.*, RGC loss took place in the eyes of rats that underwent optic nerve (ON) crush after 8 days (169). Huang *et al.* performed immunoblot analysis on rat

eyes that underwent ON crush at three time points; and no truncated CaN A was observed. In order to prove that CaN's contribution to RGC death and ON damage, Huang *et al.* compared the level of RGC death and ON damage between experimental glaucoma and experimental glaucoma with CaN inhibitor administrated (164). The immunosuppressant drug FK506 was used to inhibit CaN's activity (170). RGC loss after 10 days of elevated IOP was estimated as $33.6 \pm 2\%$ of RGC. After oral treatment with FK506, there was only a $16.7 \pm 3\%$ loss of RGC. In a pilot experiment, they assessed the degree of ON damage after 10 days of elevated IOP with/without FK506 treatment (164). A significant ON preservation was observed with FK506 treatment.

Furthermore, they examined the mechanism which led to BAD dephosphorylation and mitochondria release of Cyt *c*; and by which mechanism CaN inhibition resulted in the preservation of RGC. Phosphorylated BAD (pBAD) is inactive, while dephosphorylated Bad is active and targeted to mitochondria, where it cause cell death (166). Huang *et al.* assessed the level of pBAD in retinal cell cytoplasm from eyes with/without elevated IOP using a pBAD-specific antibody. The level of pBAD is significantly reduced in eyes with high IOP. Treatment with FK506 significantly increased the level of pBAD in eyes with high IOP. They went further to measure the levels of Cyt *c* in the cytoplasm and the mitochondria in eyes with high IOP. In eyes with high IOP, level of Cyt *c* was increased in the cytoplasm and decreased in the mitochondria; also, FK506 was able to inhibit the translocation of Cyt *c* from mitochondria to cytoplasm.

As a result, in the experimental glaucoma, CaN is cleaved into a constitutively active form; the CaN activation further enhanced pBad dephosphorylation and Cyt *c* release which

both contribute to the apoptotic RGC death. This study has revealed the molecular events that correlated to RGC death in experimental glaucoma. It will contribute to the future investigation of the biochemical mechanisms of glaucoma in patients.

1.3.2 In lower eukaryotes

A number of studies have revealed that CaN plays various roles in lower eukaryotic organisms (171, 172). For instance, CaN is involved in cell cycle progression through G1/S1 nuclear division, polarized growth and proper septation in *Aspergillus nidulans* (173, 174). It is also playing a role in differentiation and stalk cell/spore formation in *Dictyostelium discoideum* (175). In addition, CaN has been linked to virulence, pH and CO₂ homeostasis, temperature-sensitive growth and resistance to Li⁺ in *Cryptococcus neoformans* (176).

1.3.3 In plants

The first evidence that demonstrated a plant homolog of CaN was provided by using immunosuppressant drugs cyclosporine (CsA) and Tacrolimus (FK506) (177). For instance, CsA and FK506 blocked Ca²⁺-dependent inactivation of K⁺ channels in *Vicia faba* while a constitutively active form of CaN inhibited K⁺ channel activity. To date, there is no CaN purified from plant tissue. There is no gene that has been cloned for CaN from plant either. The proteins that are closely related to CaN are two EF-hand Ca²⁺-binding proteins: SOS3 proteins and AtCBL proteins (178, 179). Studies on these proteins indicate that salt stress in plants might be regulated by Ca²⁺-dependent signaling pathways (63).

1.4 CaN in Eye Tissues

The roles of CaN in other organs have implicated its potential functions in eye tissues. Our laboratory has reported that CaN is present in all eye tissues, although the activity and protein expression (including CaN A α and CaN A β isoforms) varied (180). The highest levels of CaN phosphatase activity and protein expression were observed in retina, optic nerve and cornea (180). The implications from this study expanded the insights of CaN's potential roles in eye tissues.

The presence of both isoforms of CaN A suggests a possibility that CaN might be involved in immunoregulation since CaN A β is predominantly expressed in lymphoid cells and is responsible for mediation of the immune response (181). Regarding to corneal transplantation, expression of CaN and FasL in cornea will be an important factor in determining survival of grafts since CaN is found to be necessary for the expression of FasL (164).

2.0 RATIONALE

CaN is observed in chick retina (183), in the developing and mature mammalian retina (184) and bovine eye tissues (180). In addition to the potential pathway for regulation by CaN in cornea, the response of retinal photoreceptor cells to light is another potential pathway for regulation by CaN. During the photoreceptor cycle a series of reactions result in a net decrease of cGMP concentrations in photoreceptors, causing an inactivation of cGMP-gated cation channels that allows the photoreceptor to extrude ions and return to a resting potential (185). One of the ions regulated in this process is Ca^{2+} . Intracellular Ca^{2+} concentrations in photoreceptors are also mediated by release from intracellular stores by the interaction of inositol triphosphate (IP3) (186, 187) with its receptor.

Ca^{2+} concentrations in photoreceptors could also be regulated by CaN. The pathways involving CaN could suppress the amplitude of Ca^{2+} oscillations in photoreceptors facilitating a more rapid transition from the light- to the dark-adapted photoreceptor (Figure 5). Such a possible pathway could account for the relatively high amounts of CaN demonstrated in the retina.

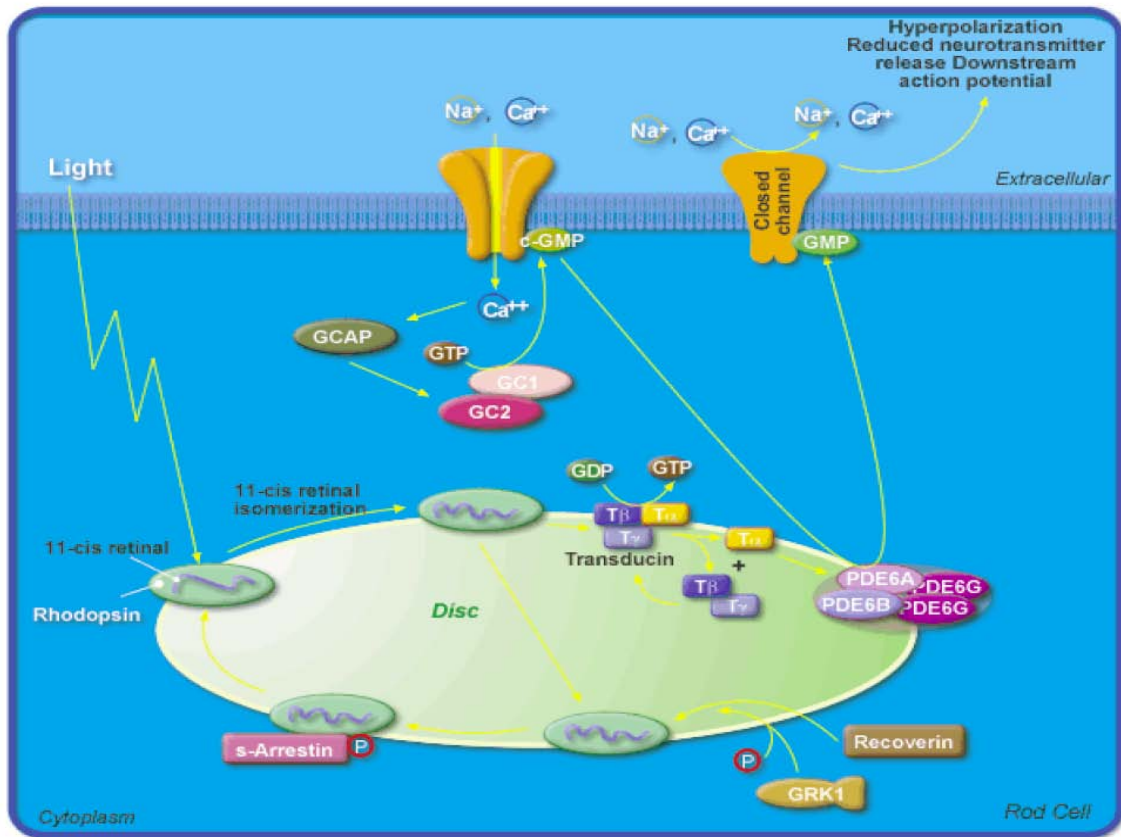


Figure 5. Visual signal transduction pathway. Figure is adapted from the “Biocarta” Web Site.
http://www.biocarta.com/pathfiles/h_rhodopsinPathway.asp (197).

3.0 SPECIFIC OBJECTIVE

- To clone bovine eye retina CaN A and B subunits.
- The biochemical characterization of recombinant CaN A subunit.
- To analyze the domain organization of recombinant CaN A subunit.

4.0 MATERIALS AND METHODS

4.1 Materials

Bovine eyes were obtained from a local slaughter house. They were transferred to the laboratory on ice and stored at -70 °C until use. The expression vector pQE9 was purchased from Qiagen, Canada. Restriction endonucleases and DNA modifying enzymes were purchased from Invitrogen, Canada. Ligation Pack was purchased from New England Biolabs, USA. Nitrocellulose sheets were purchased from Bio-Rad Laboratories, Canada. Protein markers were purchased from Invitrogen, Canada. General analytical grade laboratory chemicals were purchased from various commercial sources as listed in table 1.

4.1.1 Bacterial strains and growth media

For cloning purpose, QIAGEN EZ competent cells were purchased from QIAGEN, Canada. The genotype of QIAGEN EZ competent cells is [F[']::Tn10(Tc^r) proA⁺B⁺ lacIqZΔM15] recA1 end A1 hsdR17 (rk12⁻mk12⁺) lacglnV44 thi-1 gyrA96 relA1. For expression of CaN, bacterial strain, *Escherichia coli* M15[pREP4], was used (QIAGEN, Canada). The cells were grown in Luria Broth (LB) containing 1.0% (w/v) bacto-tryptone, 0.5% (w/v) bacto-yeast extract and 1.0% (w/v) NaCl, pH 7.0, per liter. The LB dehydrated ingredients were dissolved in deionized water and autoclaved for 20 min. at 120 °C. Filter sterilized ampicillin was also added to autoclaved LB in a final concentration of 100 µg/mL.

4.1.2 Plasmids and vectors

For cloning of CaN, pDrive cloning vector was used (QIAGEN, Canada). It contains the following elements: Multiple cloning site, LacZ α-peptide, T7 RNA polymerase promoter,

T7 transcription start, SP6 RNA polymerase promoter, SP6 transcription start, ampicillin resistant gene, kanamycin resistant gene, pUC origin, phage f1 origin, primer binding sites, M13 forward, M15 forward, M13 reverse, T7 promoter primer, and SP6 promoter primer sites.

For overexpression of CaN, pQE9 expression vector was used (QIAGEN, Canada). It provides high level expression of 6xHis-tagged proteins in *E.coli*. pQE plasmids were derived from plasmids pDS56/RBSII and pDS781/RBSII-DHFRS. It contains the following elements: multiple cloning site, T5 promoter, *lac* operator, ribosome binding site, His-tag sequence, stop codons, Col E1 origin of replication, and ampicillin resistant gene.

4.2 Methods

All methods were conformed to the Guide for the Care and Use of Laboratory Animals described and approved by the University of Saskatchewan, Saskatchewan, Canada.

4.2.1 Production of CaN antibody

A polyclonal antibody against CaN was raised in New Zealand white rabbits as described previously (180). Briefly, multiple subcutaneous injections of 50 µg bovine brain CaN emulsified with complete Freund's adjuvant (CFA) were given at multiple sites. The first blood samples were obtained on day 36, and two boosters were given with the same

Table 1. Chemicals and reagents used during current study.

Reagents and chemicals	Company, Country	Catalog number
Tris base	Invitrogen, Canada	15504-020
NaCl	Sigma-Aldrich, USA	7647-14-5
Imidazole	BDH laboratory supplies, Canada	EM-IX0005-2
IPTG	Invitrogen, Canada	15529-019
<i>p</i> NPP	Sigma-Aldrich, USA	698999-89-2
2-mercaptoethanol	Sigma-Aldrich, USA	60-24-2
Western lightning chemiluminescence reagent plus	PerkinElmer LAS, Inc., USA	NEL104
Bio-Rad Protein Assay	Bio-Rad, USA	500-0006
EGTA	Sigma-Aldrich, USA	67-42-5
SDS	BDH chemicals, Canada	EM-7910
X-GAL	Invitrogen, Canada	15520-018
Ampicillin sodium salt	Invitrogen, Canada	11593-019
Goat Anti-Rabbit IgG(H+L)-HRP conjugate	Bio-Rad, USA	170-6515
Lysozyme	Sigma-Aldrich, USA	12650-88-3
BenchMark protein ladder	Invitrogen, Canada	10747-012
BenchMark pre-stained protein ladder	Invitrogen, Canada	10748-010
1 kb DNA Ladder	Invitrogen, Canada	15615-016
[γ -32P]ATP	PerkinElmer LAS, Inc., USA	BLU002
<i>Bam</i> HI	Invitrogen, Canada	15201-023
<i>Hind</i> III	Invitrogen, Canada	15207-020
Acrylamide	Invitrogen, Canada	15512-023
Agarose	Invitrogen, Canada	15510-027
Nitrocellulose Membrane	Bio-Rad, USA	162-0115
X-Omat Blue XB-1	Kodak, USA	1776699
Blotting Grade Blocker Non-Fat Dry Milk	Bio-Rad, USA	170-6404
Bovine Serum Albumin	Sigma-Aldrich, USA	9048-46-8

amount of antigen in CFA, one on day 37 and the other on day 56. Subsequent blood samples were obtained on days 55 and 60. The sera were separated from the blood samples and stored at -20 °C until use. The antibody titer was determined by using ELISA (188). The immunoglobulins were purified through a protein A-Sepharose-4B column, which was pre-equilibrated with 100 mM Tris-HCl, pH 8.0, followed by elution of bound IgG with 100 mM glycine, pH 2.5. The pH was adjusted to 8.0 with 1 M Tris immediately after elution. The immunoglobulin was dialyzed against phosphate-buffer saline (PBS) overnight. The antibody was demonstrated to be specific for CaN. The dialyzed sample was stored at -70 °C until use.

4.2.2 Preparation of CaM

Bovine brain CaM was purified by using a phenyl-Sepharose column as described by Gopalakrishna and Anderson (189). It was further purified by using a Sephacryl S-200 gel filtration column which was pre-equilibrated with Buffer A (20 mM Tris-HCl, 1 mM magnesium acetate, 1 mM imidazole, pH 7.0, 10 mM 2-mercaptoethanol) containing 0.01 mM Ca^{2+} and 0.1 M NaCl.

4.2.3 Preparation of cAMP-PK

Bovine heart cAMP-PK was purified to homogeneity as described by Demaille *et al.* (190). Bovine heart was homogenized in 2.5 vols of 4 mM EDTA, 0.2 mM mercaptoethanol, pH 7.0 and centrifugated at 7,000 X g for 30 minutes. The supernatant was filtered through glass wool and pH was adjusted to 7.6. The clear supernatant was added to DEAE-Sepharose 6B Cl which was pre-equilibrated with buffer A (50 mM Tric-HCl, pH 7.6, 10 mM NaCl, 4 mM EDTA, 0.2 mM mercaptoethanol) and mixed overnight. Filtered the suspension on sintered glass funnel and washed the DEAE-Sepharose 6B Cl thoroughly with buffer A.

DEAE-Sepharose 6B Cl column was prepared and equilibrated with buffer A. The washed from sintered glass funnel was loaded to DEAE-Sepharose 6B Cl column and eluted with buffer A containing 0.5 M NaCl. $(\text{NH}_4)_2\text{SO}_4$ was added to the eluent in a final concentration of 176 g/L and mixed for 1 hour. The suspension was centrifuged at 11,000 X g for 45 minutes. $(\text{NH}_4)_2\text{SO}_4$ (314 g/L) was added to the supernatant and adjusted pH to 7.0 and mixed for 1 hour. Then, centrifuged at 11,000 X g for 45 minutes and redissolved the pellet in minimum volume of buffer B (30 mM K_2HPO_4 , pH 7.0, 1 mM EDTA, 0.2 mM mercaptoethanol) and dialyzed overnight against buffer B. Then, the dialysed supernatant was adjusted pH 6.1. Then, resulted supernatant was mixed with CM Sephadex C-50 which was pre-equilibrated with buffer C (30 mM K_2HPO_4 , pH 6.1, 1 mM EDTA, 0.2 mM mercaptoethanol). cAMP was added to filtrate to a final concentration of 10 μM and incubated for 1 hour with 60 mL CM Sephadex C-50 pre-equilibrated with buffer C. Then, filtered on a Buchner funnel and washed with 12 volume of buffer C. The resin was resuspended in a minimum volume of buffer C and packed in a column and washed with 50 mL of buffer C. Proteins were eluted with buffer C containing 1.0 M KCl and collected ~5.0 mL fraction at 25 mL/hour. Pooled A280 absorbing fractions and concentrated to minimum volume. Centrifuged at 30,000 rpm using 75 Ti rotor for 30 minutes. Supernatant was loaded on a column of Sephacryl S-200 which was equilibrated with buffer D (30 mM K_2HPO_4 , pH 7.0, 1 mM EDTA, 2 mM mercaptoethanol and 0.15 M KCl). The fractions were collected and analyzed for cAMP-PK activity. The highest enzyme activity fractions were pooled and stored at -80 °C for further analysis.

4.2.4 Molecular cloning of bovine retina CaN A and CaN B

General cloning techniques were carried out essentially as described by Sambrook *et al.* (191). Total RNA was prepared from bovine retina muscle using the RNeasy mini kit (Qiagen). Reverse transcription-polymerase chain reactions (RT-PCR) were carried out using sense and antisense oligonucleotide primers specific for CaN A and B. Primers were designed based on the bovine brain CaN A (accession number U33868) and CaN B (accession number NM174583) sequences. The retina CaN A and B cDNAs were amplified by employing PCR using GeneAmp PCR System 9600 (Perkin–Elmer, USA) using two primers with restriction sites (underlined) for *Bam*HI and *Hind*III using one-step RT-PCR (Qiagen, Canada) (Table 2). The details of primer sequences were shown in the table 3. The amplified PCR product was purified from agarose gels using a QIAquick gel extraction kit (Qiagen, Canada). Resulting DNA fragments (CaN A and CaN B) were cloned into the pCR-Zero Blunt vector using the pCR-TOPO cloning kit (Invitrogen, Canada). The recombinant plasmid (BT-CaN-A- Zero Blunt) was subjected to DNA sequencing to check the nucleotide sequences of the amplified genes. The complete nucleotide sequence was determined by the dideoxy chain termination method using a DNA sequencer (Applied Biosystem Model 310A). The CaN A gene was excised with *Bam*HI and *Hind*III and ligated into the expression vector pQE9 (previously digested with same restriction enzymes), resulting in plasmid BT-CaN A-pQE9. Similarly, CaN B was cloned as described above using CaN B specific primers.

4.2.5 Expression and Purification of recombinant retinal CaN A and CaN B

For the expression of recombinant protein, BT-CaN-A-pQE9 was transformed into *E. coli* M15 (pREP4) competent cells and grown in LB medium (100 µg/ml ampicillin) at 37 °C with shaking. After reaching an optical density of 0.4–0.6 at 600 nm the production of target

protein was induced by the addition of isopropyl-beta-D-thiogalactopyranoside (IPTG) (to 1 mM). Cells were induced for 5 hour and were harvested by centrifugation (8000 X *g*, 20 minutes at 4 °C). Cells were suspended in lysis buffer (50 mM Tris-HCl, pH 8.0, 300 mM NaCl, 10 mM imidazole) and were lysed by addition of lysozyme to a final concentration of 1 mg/ml on ice for 30 minutes. This was followed by sonication in a Sonics VibraCell Sonicator, for 12 X 15 s bursts. The lysate was cleared by centrifugation (14,000 X *g*, 30 minutes at 4 °C) and loaded on to a Ni-NTA agarose column, equilibrated with lysis buffer. The column was washed until no protein with wash buffer (50 mM Tris-HCl, pH 8.0, 300 mM NaCl and 20 mM imidazole), and the bound protein was eluted from the column using elution buffer (50 mM Tris-HCl, pH 8.0, 300 mM NaCl and 250 mM imidazole). Similarly, the CaN B was expressed and purified as described above.

4.2.6 CaN assay

CaN activity was assayed using pNPP as a substrate (192). The reaction mixture consists of 50 mM Tris-HCl, pH 7.0, 5 µg CaM, 3.4 mM pNPP, 15 µg CaN A and 10 µg CaN B in total volume of 1.0 ml. Ca^{2+} -independent activity of CaN was determined under identical conditions in the presence of 5 mM EGTA. The reaction was incubated at 30 °C for 30 minutes. The reaction was initiated by the addition of pNPP and terminated by the addition of 75 mM K_2HPO_4 . The pNPP hydrolysis was quantified by the increase of absorbance at 405 nm. One unit of phosphatase activity was defined as the amount of dephosphorylation

Table 2. PCR conditions of CaN Subunits

cDNA of CaN subunits	RT-PCR conditions
CaN A	50 °C for 30 minutes, 95 °C for 15 minutes, 35 cycles of (94 °C for 15 seconds, 53 °C for 30 seconds, 72 °C for 2 minutes), 72 °C for 10 minutes.
CaN B	50 °C for 30 minutes, 95 °C for 15 minutes, 35 cycles of (94 °C for 15 seconds, 51 °C for 30 seconds, 72 °C for 30 seconds), 72 °C for 10 minutes.

Table 3. Primers for RT-PCR of WT CaN and Various Mutants.

CaN subunit	RT-PCR primers
CaN A	Forward: 5'-GGATCCATGTCCGAGCCCAAGGCAAT-3' Reverse: 5'-AAGCTTTCCTGATATTGCTGCTATTACTGCC-3'
Δ1 CaN A97-511	Forward: 5'-GGATCCGACTTGATGAACTCTTTGAAGTGGGG-3' Reverse: 5'-AAGCTTTCCTGATATTGCTGCTATTACTGCC-3'
Δ2 CaNA201-511	Forward: 5'-GGATCCGGCGGTTTGTCTCCAGAGATAAACA-3' Reverse: 5'-AAGCTTTCCTGATATTGCTGCTATTACTGCC-3'
Δ3 CaN A1-456	Forward: 5'-GGATCCATGTCCGAGCCCAAGGCAAT-3' Reverse: 5'-AAGCTTCCCAACAAACGGCAGGGAC-3'
Δ4 CaN A1-406	Forward: 5'-GGATCCATGTCCGAGCCCAAGGCAAT-3' Reverse: 5'-AAGCTTCATCTTGCCAATGGCTCGGA-3'
Δ5 CaN A1-358	Forward: 5'-GGATCCATGTCCGAGCCCAAGGCAAT-3' Reverse: 5'-AAGCTTTAAGCCCTTGGCCTCCTCGA-3'
CaN B	Forward: 5'-GGATCCATGGGAAATGAGGCAAGTTATCCTTTG-3' Reverse: 5'-AAGCTTTCACACGTCTACCACCATCTTTTTGTG-3'

resulting in an optical density of 0.1 at 30 °C after 30 minutes incubation.

4.2.7 Effects of metal ions on CaN activity

To determine the effect of metal ions on CaN phosphatase activity, the recombinant retinal CaN incubated with 1 mM Ni^{2+} , Fe^{2+} , Mn^{2+} or Zn^{2+} in the reaction mixture as described in 4.2.6.

4.2.8 Deletion analysis

All deletion constructs were generated by PCR using specific oligonucleotide primers (Table 3) and standard methodologies as detailed previously. All constructs generated by PCR were sequenced to eliminate potential mutations incorporated during the PCR. All mutants were expressed and purified as described above.

4.2.9 Phosphorylation and dephosphorylation of histone

Histones were phosphorylated essentially as previously described by Sharma (193). The standard reaction mixture consists of 100 mM Tris-HCl, pH 7.0, 5 mM MgCl_2 , 5 mM 2-mercaptoethanol, histone (2 mg/mL), 0.1 mM $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (150–200 cpm/pmol) and catalytic subunit of cAMP-PK (20 $\mu\text{g/mL}$) in the presence of 0.1 mM EGTA. After incubation for 60 minutes an aliquot was taken for the determination of phosphate incorporation. The remaining sample of phosphorylated histone will be dialyzed overnight with several changes against 20 mM Tris-HCl, pH 7.0 and 10% sucrose to remove the unreacted $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. For dephosphorylation of histone, the phosphorylated histone (0.2 mg/mL) was incubated at 30 °C in a reaction mixture containing 100 mM Tris-HCl, pH 7.0, 5 mM 2-mercaptoethanol, and CaM (100 $\mu\text{g/mL}$) with the addition of retina WT CaN A (15 $\mu\text{g/mL}$) or various truncated mutants (15 $\mu\text{g/mL}$) and retina CaN B (10 $\mu\text{g/mL}$) incubated with 0.1 mM Ni^{2+} . The retinal

CaN A or various truncated mutants and CaN B were incubated with 1 mM Ni^{2+} at room temperature for 1 hour prior to the incubation of phosphatase reaction.

4.2.10 SDS-PAGE and Western blot analysis

Purified retinal recombinant CaN A and B were separated on 10% and 15% SDS-PAGE, respectively, according to the procedure described by Laemmli (194). The bacterially expressed retina CaN A and B were transferred to nitrocellulose using the immunoblot method of Towbin *et al* (195). The blot was incubated first with the CaN specific polyclonal antibody at a dilution of 1:1000, washed and probed with an anti-rabbit IgG horseradish peroxidase conjugate (Bio-Rad) diluted 1:2000. Membranes were incubated in chemiluminescence reagent (NEN Life Science Products, USA) and exposed to Kodak X-OMAT Blue XB-1 film for the detection of CaN.

4.2.11 Other methods

Protein concentrations were measured by the method of Bradford (196) using bovine serum albumin as a standard. The nucleotide and amino acid sequences were evaluated using the MacVector (version 7) computer program.

4.2.12 Statistical analysis

All data were analyzed as the mean $\pm$ S.E. The difference between the mean values was tested by the two-tailed Student's *t*-test for statistical significance.

5.0 RESULTS AND DISCUSSIONS

5.1 Cloning of CaN A and CaN B

In order to characterize the biochemical and structural properties of bovine retina CaN, molecular cloning techniques were employed. Total RNA was isolated from bovine retina cells. Two pairs of DNA primers were designed based on bovine brain CaN A and CaN B subunits. Then, the cDNA copies of CaN A and CaN B were obtained by using a one-step RT-PCR kit. The retinal CaN A cDNA and CaN B cDNAs were subjected to 1% and 2% agarose gel electrophoresis, respectively, to visualize the size and purity of the PCR products (Figure 6). A single band was observed for each subunit: CaN A and CaN B, 1500 bp and 500 bp, respectively (Figure 6).

The cDNA copies of the two subunits were purified from agarose gel using a QIAquick gel extraction kit. Resulting DNA fragments were cloned into the pCR-Zero Blunt vector using pCR-TOPO cloning kit. The recombinant plasmid BT-CaN A-Zero Blunt was subjected to DNA sequencing to confirm the nucleotide sequence of retinal CaN A (Figure 7). The complete sequence of CaN A specifies 1536 nucleotides. It was translated into peptide sequence and subjected to comparison with CaN A peptide sequences from other sources or species (Figure 8).

Bovine eye CaN A has 10 amino acids deletion: A-T-V-E-A-I-E-A-D-E-A, between 447 and 458 compared to bovine brain CaN A. There was also one amino acid difference at position 367 (aspartic acid → asparagine) between bovine eye CaN A and bovine brain CaN A (Figure 8). However, it was found that bovine eye CaN A was sharing 100% identity with

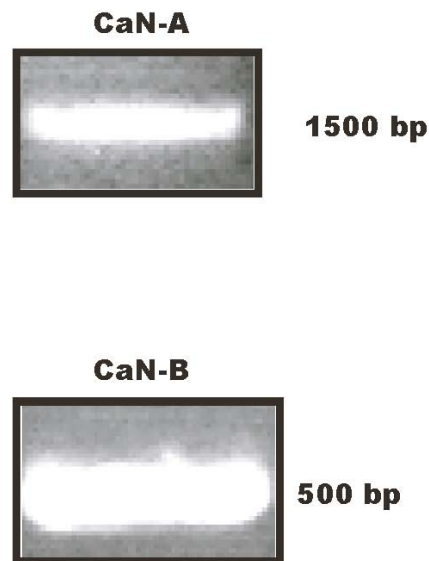


Figure 6. RT-PCR amplification of CaN A from bovine eye retina tissues. The CaN A and B cDNAs were amplified by employing PCR using GeneAmp PCR System 9600 (Perkin–Elmer, USA) using two primers with restriction sites (underlined) for *Bam*HI and *Hind*III with the nucleotide sequences of GGA TCC ATG TCC GAG CCCAAG GCAAT and AAG CTT TCA CTG GAT ATT GCT GCT ATT ACT GCC for CaN A, and GGA TCC ATG GGA AAT GAG GCA AGT TAT CCT TTG and AAG CTT TCA CAC GTC TAC CAC CAT CTT TTT GTG for CaN B. The resulting PCR products were resolved by electrophoresis on a 1 or 2% agarose gel and then visualized by ethidium bromide staining of the gel.

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      10      20      30      40      50      60      70      80      90
ATGTCGAGCCCAAGGCAATTGATCCCAAG TTGTGACGACCGACAGGGTGGTGAAAGCT GTCCCATTTCTCCCAAGTCACCGGCTTACG
M S E P K A I D P K L S T T D R V V K A V P F P P S H R L T>

      100     110     120     130     140     150     160     170     180
GCAAAAGAAGTGTGTTGATAATGATGGGAAA CCTCTGTGTGGATATCTTAAAGGCACATCTC ATGAAGGAGGGCAGGCTCGAAGAGACTGTC
A K E V F D N D G K P R V D I L K A H L M K E G R L E E T V>

      190     200     210     220     230     240     250     260     270
GCATTGAGATAATAACAGAGGGGCTTCA ATTCTTCGACAGGAAAAAACTTGTCTGGAT ATTGATGCTCCAGTCACAGITTTGTGGGGAC
A L R I I T E G A S I L R Q E K N L L D I D A P V T V C G D>

      280     290     300     310     320     330     340     350     360
ATCCATGGACAATTCTTTGACTTGATGAAA CTCTTTGAAGTGGGGGATCTCTGCGCAAC ACTCGCTACCTCTTCTTAGGGGACTATGTT
I H G Q F F D L M K L F E V G G S P A N T R Y L F L G D Y V>

      370     380     390     400     410     420     430     440     450
GACAGAGGGTACTTCAGTATCGAATGTGTG CTGTATTGTGTGGGCTTTGAAAATTCTTTAC CCGAAAACACTGTTTTTACTTCTGTGGAAAT
D R G Y F S I E C V L Y L W A L K I L Y P K T L F L L R G N>

      460     470     480     490     500     510     520     530     540
CATGAATGTAGACATCTAACAGAGTATTTC ACATTAAACAAGAATGTAAATAAAGTAT TCAGAACGCGTATATGATGCGTCATGGAT
H E C R H L T E Y F T F K Q E C K I K Y S E R V Y D A C M D>

      550     560     570     580     590     600     610     620     630
GCCTTGACTGCGCTGCGCCCTGGCTGCCCTG ATGAACGACAGTTCCTGTGTGTACACGGC GGTTCGTCTCCAGAGATAAACACTTTAGAT
A F D C L P L A A L M N Q Q F L C V H G G L S P E I N T L D>

      640     650     660     670     680     690     700     710     720
GATATCAGAAAATTAGACCGATTCAAAGAA CCACCTGCTTATGGACCTATGTGTGATATC CTGTGCTCAGACCGCTGGAAGATTTTGA
D I R K L D R F K E P P A Y G P M C D I L W S D P L E D F G>

      730     740     750     760     770     780     790     800     810
AACGAGAGACTCAGGAACATTCTACTCTAC AACACAGTCAGGGGGTGTTCATACTTCTAC AGTTACCCGGCTGTATGTGAATTCTTGCAG
N E K T Q E H F T H N T V R G C S Y F Y S Y P A V C E F L Q>

      820     830     840     850     860     870     880     890     900
CACAAATCTGTATCTATCTCCGAGCT CATGAAGCCCAAGATGCGGGTACCGCATG TACAGGAAAAGCCAAACACAGGCTTCCCC
H N N L L S I L R A H E A Q D A G Y R M Y R K S Q T T G F P>

      910     920     930     940     950     960     970     980     990
TCTCTCATTACAATCTTTTCAGCACCAAAAT TACTTAGATGTATACAATAACAAAGCTGCA GTATTGAAGTATGAGAACAATGTCTATGAAT
S L I T I F S A P N Y L D V Y N N K A A V L K Y E N N V M N>

     1000    1010    1020    1030    1040    1050    1060    1070    1080
ATCAGGCAATTCAACTGTTCTCTCATCCA TACTGGCTTCCAAATTTCAATGGATGTTTTT ACCTGGTCCCTGCGGTTTGTGGGGAAAAA
I R Q F N C S P H P Y W L P N F M D V F T W S L P F V G E K>

     1090    1100    1110    1120    1130    1140    1150    1160    1170
GTGACTGAGATGCTGGTAGATGTCTCAAC ATATGCTCAGACGATGAAGTACGGGTGAGAA GAAGATGGATTTGATGGAGCAACGGCTGCA
V T E M L V D V L N I C S D D E L G S E E D G F D G A T A A>

     1180    1190    1200    1210    1220    1230    1240    1250    1260
GCCAGGAAGGAGGTGATAAGAAATAAGATC CGAGCCATTGGCAAGATGGCCAGAGTGTTT TCGGTCTCAGAGAGAGAGTGAAGTGTG
A R K E V I R N K I R A I G K M A R V F S V L R E E S E S V>

     1270    1280    1290    1300    1310    1320    1330    1340    1350
CTGACGCTGAAAGGCTTGACGCCACAGGC ATGCTCCCGACGCGCGTCTTTCTGGAGGG AAACAAACCTGCAAGCGCTATCAAAAGGA
L T L K G L T P T G M L P S G V L S G G K Q T L Q S A I K G>

     1360    1370    1380    1390    1400    1410    1420    1430    1440
TTTTCAACACACATAAGATCACTAGCTTC GAGGAGGCCAAGGGCTTAGACCGAATTAAT GAGAGGATGCCACCTGCCAGAGATGCCATG
F S P Q H K I T S F E E A K G L D R I N E R M P P R R D A M>

     1450    1460    1470    1480    1490    1500    1510    1520    1530
CCCTCTGATGCCAACCTTAACCTCAATCAAC AAGGCTCTCGCCTCAGAGACTAACGGCACG GACAGCAATGGCAGTAATAGCAGCAATATC
P S D A N L N S I N K A L A S E T N G T D S N G S N S S N I>

CAGTGA
Q *>

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Figure 7. Nucleotide sequence of the coding region of bovine eye retina CaN A and the encoded 511 amino acid residues.



Figure 8. Comparison of the amino acid sequence of different species of CaN A. The multiple alignment was generated using the ClustalW program. The conservation is represented by the following colors: primary (red, 90-100%), secondary (blue 70-89 %), tertiary (green 50-69 %), and quaternary (yellow, 30-49 %). The deduced amino acid sequence of bovine eye CaN A was aligned with *Bos taurus* cardiac CaN A, GenBank ABB22788; *Bos taurus* brain CaN A, GenBank NP7777212; *H. sapiens* brain CaN A, GenBank NM000944; *R. norvegicus* brain CaN A, GenBank NM01704 -1; *M. musculus* brain CaN A, GenBank NP032939.

bovine heart CaN A. Despite the 10 amino acids deletion, there were three amino acid substitutions: threonine → serine at position 59, aspartic acid → asparagine at position 367, alanine → threonine at position 504, between bovine eye CaN A and human CaN A (Figure 8). There were three amino acid substitutions: threonine → serine at position 59, glutamic acid → aspartic acid at position 267, aspartic acid → asparagine at position 367, between bovine eye CaN A and rat CaN A, addition to the 10 amino acids deletion (Figure 8). Rat brain and mouse brain CaN A share 100% sequence identity. The results suggest that CaN A sequence is highly conserved in mammals. It is highly possible that the functionality and physiological roles of CaN A among these species are more or less the same. CaN A might play important roles in biological systems since it is widely expressed in various species and tissues.

5.2 Expression and purification of CaN A and CaN B

CaN A DNA was excised by using endonuclease digestion with *Bam*HI and *Hind*III. The gene was then ligated into an expression vector pQE9, resulting in plasmid BT-CaN A-pQE9. CaN B was cloned into pQE9 in the same procedure. To express both subunits, the resulting plasmids were transformed into *E. coli* M13 (pREP4) separately. The expressions of both proteins were induced by using IPTG as described in section of Materials and Methods. The recombinant retinal CaN A was linked to a 6X Histidine tail at its NH₂-terminus. The purification of the recombinant protein was carried out by applying crude lysate to Ni-NTA agarose column. The His-tagged protein was tightly bound with the Ni-coated column while other proteins and impurities were flow through and rinsed out from the column. Thereby,

this single step purification was efficient enough to obtain the purified CaN A. Retinal CaN B recombinant protein was also purified in the same procedure.

In order to judge the purity and estimate the sizes of CaN A and CaN B, both proteins were subjected to 10% and 15% SDS-PAGE, respectively (Figure 9). CaN A was migrated with a molecular mass of approximately 60 kDa while CaN B was migrated with a molecular mass close to 20 kDa. In order to confirm the identity of visualized bands on SDS-PAGE, the bands were transferred onto a nitrocellulose membrane and incubated with polyclonal antibody raised against bovine brain CaN (Figure 10). Both CaN A and CaN B protein bands were detected by the antibody at the correct position relative to the pre-stained protein marker. Thus, both proteins were purified successfully to near homogeneity.

5.3 CaN phosphatase activity and effect of metal ions

Once both subunits were purified, the enzymatic activity of CaN was examined. CaN A has the phosphatase activity since it contains the active site. However, the association of CaN A and CaN B subunits is required to achieve its full activity. In order to study the stoichiometry ratio between the two subunits, the reconstitution assay was carried out with a constant concentration of CaN A and various concentrations of CaN B subunits (Figure 11). As a result, the CaN phosphatase activity reached maximal level when stoichiometry ratio between CaN A and CaN B was 1:2. The maximal CaN A phosphatase activity was reached when one molecule of CaN A subunit binds to two molecules of CaN B subunits. Therefore, all the subsequent phosphatase activity assays were carried out at this molar ratio. Furthermore, the effects of metal ions including nickel (Ni^{2+}), ferric (Fe^{2+}), mangness (Mn^{2+}) and zinc (Zn^{2+}) on CaN phosphatase activity were studied by using pNPP as a substrate.

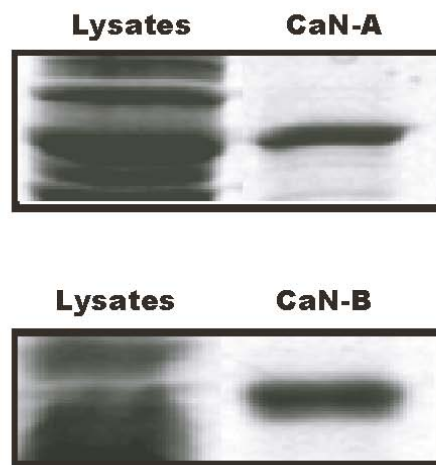


Figure 9. SDS-PAGE analysis of purified recombinant bovine eye retina CaN A and CaN B. Five microgram of purified *E. coli* expressed eye retina CaN A and B were loaded on each lane. Upper panel; lane 1, crude lysate of CaN A; lane 2, purified CaN A; and lower panel; lane 1, crude lysate of CaN B; lane 2, purified CaN B. The data presented are representative of three independent experiments.

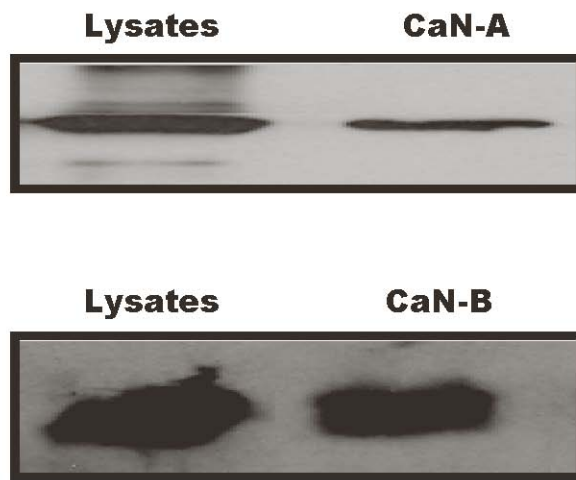


Figure 10. Western blot analysis of eye retina CaN A and B. Five microgram of purified *E. coli* expressed eye CaN A and B were immunoblotted with polyclonal anti-CaN (1:1000 dilution) as described in the Materials and Methods Section. The data presented are representative of three independent experiments.

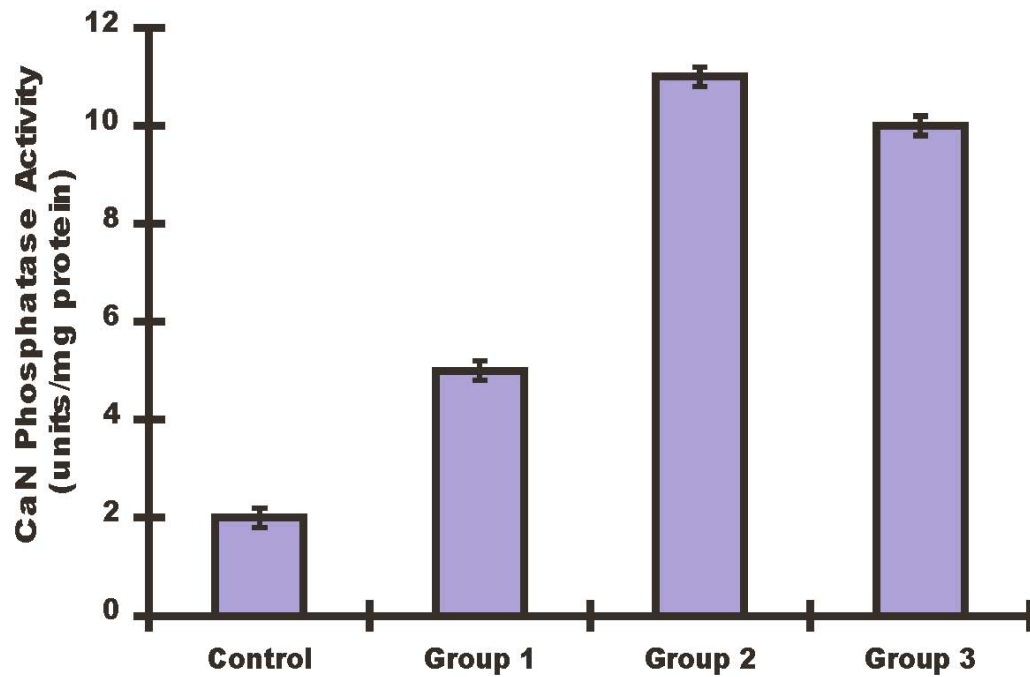


Figure 11. Reconstitution of CaN A and CaN B for CaN phosphatase activity. CaN activity was determined using pNPP as a substrate as described in the Materials and Methods Section. Control: CaN A alone; group 1, CaN A and CaN B in 1: 1 stoichiometry ; group 2, CaN A and CaN B in 1: 2 stoichiometry; group 3, CaN A and CaN B in 1: 3 stoichiometry. Statistical significance was determined using Student's *t*-test analysis. The data presented are representative of three independent experiments.

Ni^{2+} was found to be the strongest stimulator while Zn^{2+} was found to be the inhibitor of CaN phosphatase activity (Figure 12).

5.4 Construction of deletion mutants of CaN A

Five deletion mutants of CaN A were constructed, expressed and purified as described in “Materials and Methods”. Two mutants were constructed by progressively deleting the regions at NH₂-terminus of CaN A. Since the active site lies close to NH₂-terminus, the region(s) essential for its phosphatase activity can be defined (Figure 13). Three mutants were constructed by progressively deleting the regions at C-terminus. The domains which have regulatory effects on its phosphatase activity can be defined since the auto-inhibitory, CaM-binding and CaN B-binding domains are located close to C-terminus (Figure 13). The purified mutants were subjected to SDS-PAGE to confirm the purity and estimate their sizes (Figure 14). All the mutants were judged to be purified close to homogeneity. CaN A97-511 ($\Delta 1$), 49 kDa; CaN A201-511 ($\Delta 2$), 37 kDa; CaN A1-456 ($\Delta 3$), 52 kDa; CaN A1-406 ($\Delta 4$), 48 kDa; and CaN A1-358 ($\Delta 5$), 42 kDa.

5.5 CaN phosphatase activity of WT and deletion mutants

Once both the WT CaN A and the mutants were purified, they were tested for phosphatase activity assay to observe any changes due to deletion (Figure 15). WT CaN A alone showed some basal phosphatase activity increased almost 50% upon association with CaN B. However, the addition of EGTA caused inactivation of phosphatase activity in the presence of CaM. This indicates that both CaN B and CaM are essential to achieve the full activity of CaN A. CaN A97-511 ($\Delta 1$) did not display any significant difference in terms of

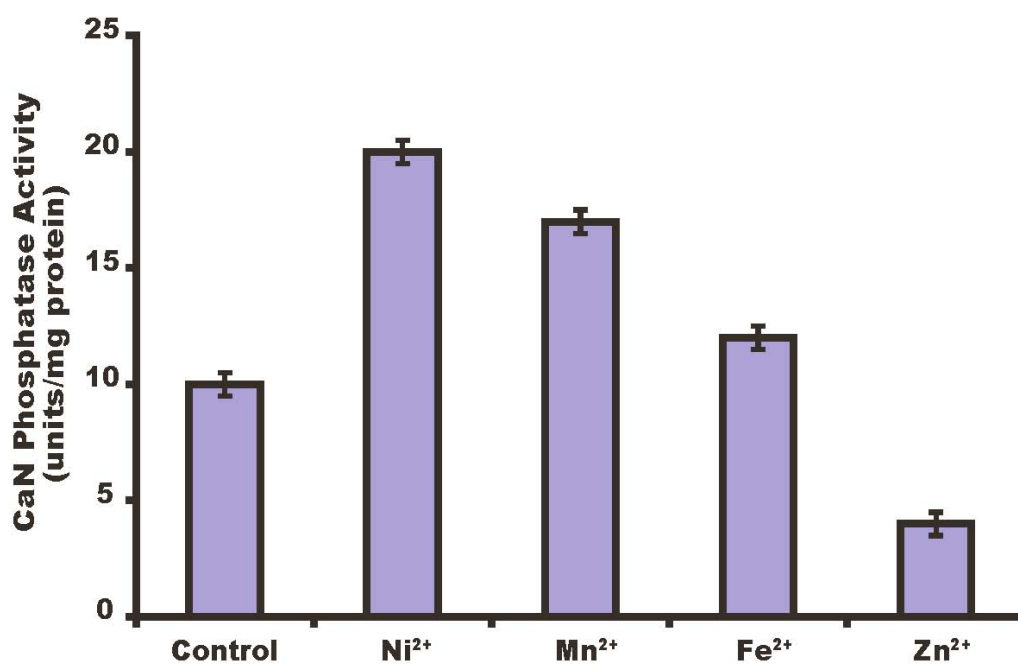


Figure 12. Effect of metal ions on bovine eye recombinant CaN phosphatase activity. CaN activity was determined using pNPP as a substrate as described in the Materials and Methods Section. The data presented are the mean of at least three independent experiments.

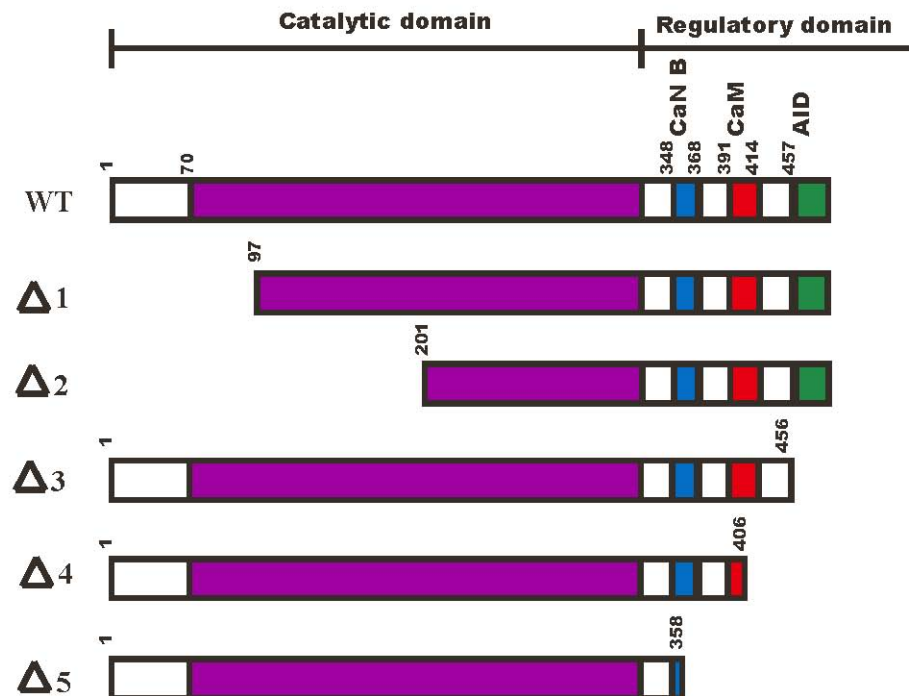


Figure 13. Schematic representation of various truncated mutants. WT, CaN A; $\Delta 1$, CaN A97–511; $\Delta 2$, CaN A201–511; $\Delta 3$, CaN A1–456; $\Delta 4$, CaN A1–406; and $\Delta 5$, CaN A1–358. Purple, catalytic domain; blue, CaN B binding domain; red, CaM binding domain; green, AID domain.

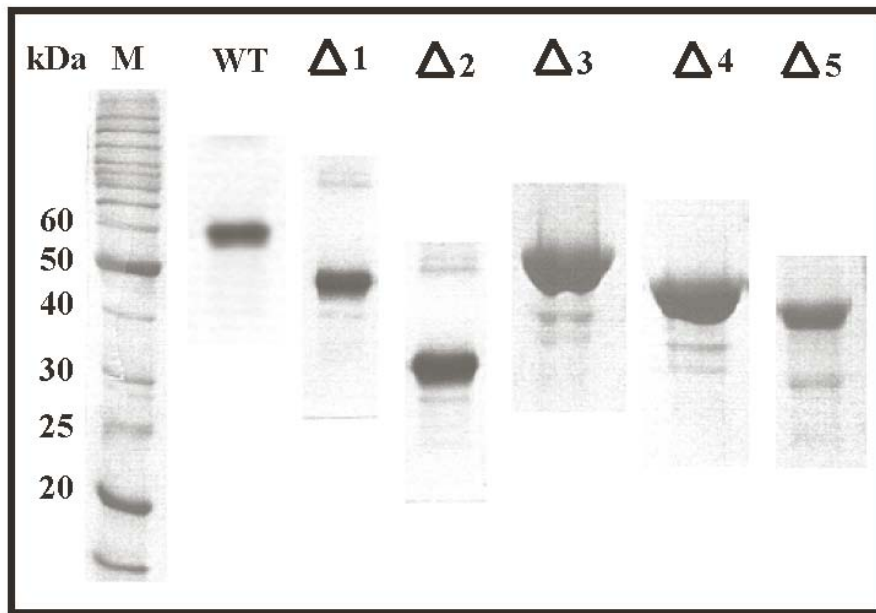


Figure 14. SDS-PAGE analysis of purified CaN A and various truncated mutants. M, molecular weight marker; WT, CaN A; $\Delta 1$, CaN A97–511; $\Delta 2$, CaN A201–511; $\Delta 3$, CaN A1–456; $\Delta 4$, CaN A1–406; and $\Delta 5$, CaN A1–358.

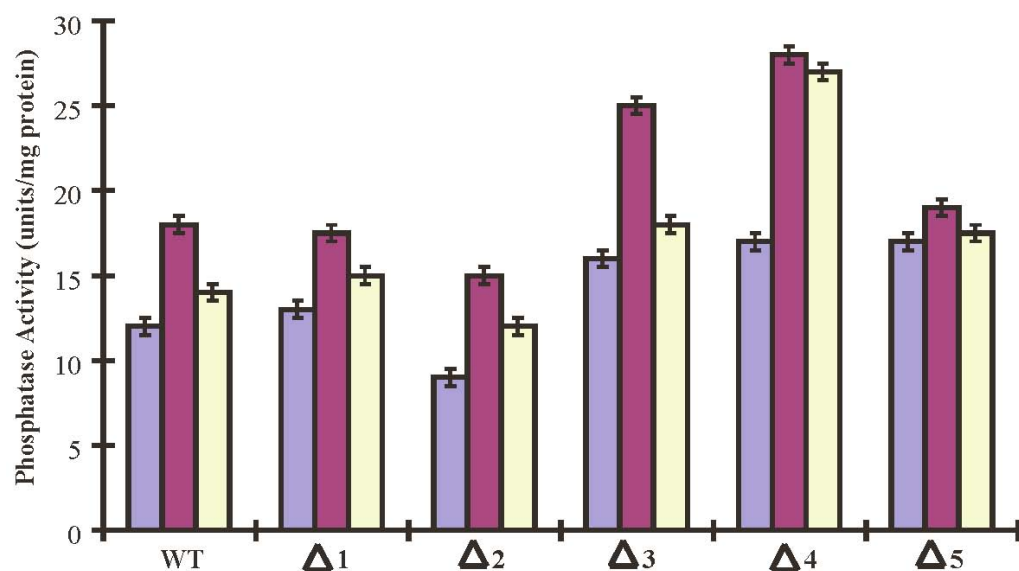


Figure 15. CaN phosphatase activity in WT and truncated CaN A mutants. WT, CaN A; Δ1, CaN A97–511; Δ2, CaN A201–511; Δ3, CaN A1–456; Δ4, CaN A1–406; and Δ5, CaN A1–358. CaN activity was determined using pNPP as a substrate as described in the Materials and Methods Section. CaN phosphatase activity was assayed in the presence of Ni^{2+} and CaM. CaN A alone (blue), CaN A and B (red). CaM-independent CaN A and CaN B phosphatase activity was also carried out in the presence of EGTA (yellow). The data presented are representative of at least three independent experiments.

basal phosphatase activity as well as CaN B- and CaM-dependence of phosphatase activity. This suggests that the deleted residues 1-97 at NH₂-terminus do not have any impact on CaN A activity. However, CaN A201-511 ($\Delta 2$) displayed reduced both basal and full activity compared to WT. This suggests that the amino acid residues 97-200 are important for CaN phosphatase activity.

Moreover, CaN A 1-456 ($\Delta 3$) displayed significant increase in both basal and full activity compared to WT. The truncated CaN A alone showed a ~30% increase compared to WT CaN A alone activity. When CaM is inactivated, CaN A1-456 still showed an equivalent phosphatase activity level compared to the full activity of WT CaN A, which needs to be activated by CaM (Figure 15). This must be due to the removal of autoinhibitory domain. Hence, binding of CaM is not required for CaN A1-456 ($\Delta 3$) to free the active site from inhibition by autoinhibitory domain. Also, previous studies have demonstrated the reduction of inhibitory effect upon one amino acid mutation at position 477 Asp to Asn in the autoinhibitory domain (199). Thus, increase of catalytic activity can be achieved by removing or disruption of autoinhibitory domain.

Furthermore, its phosphatase activity was further enhanced by nearly 40% when CaN A1-456 ($\Delta 3$) was bounded to CaM (Figure 15). This confirms that CaM is essential for the maximum activity of CaN. It also suggests the autoinhibitory domain is not the only region responsible for the inhibition of phosphatase activity. When CaM is not binding to CaN A, there might be some type of structural obstruction prevent the substrate from accessing the catalytic site. This blockage can be removed upon binding of CaM to CaN A.

It is highly possible that when CaM is not binding to CaN A, the catalytic site is inhibited by some structural constraint. Upon binding of CaM, some conformational change might have occurred in the catalytic site. In this way, the catalytic site might have become more efficient to dephosphorylate its substrate. This observation agreed with the previous study on the synergism between the CaM-binding domain and autoinhibitory domain on CaN phosphatase activity (200).

A further increase in both basal and full activity was observed from CaN A1-406 ($\Delta 4$) (Figure 15). It implies that more inhibition was removed from the phosphatase activity. The residues at 406-456 contain inhibitory effect on the active site. This observation agrees with an earlier study by Perrino (201). A second autoinhibitory domain lies within residues 420-457, which is located at the C-terminal to the CaM-binding domain (201). The second inhibitory effect could be removed when CaM binds to CaN A (201). This was also observed by inactivating CaM in the dephosphorylation by CaN A1-456 ($\Delta 3$) (Figure 15). However, the presence of EGTA (the inactivation of CaM) did not have any impact on CaN A1-406 ($\Delta 4$) activity (Figure 15). This suggests that CaM-dependence of its activity was removed along with the deleted portion from C-terminus. It also confirmed that the CaM-binding domain precedes the autoinhibitory domain in CaN A (201, 202). In contrast, it is not the case in other CaM-dependent proteins such as CaMPKs and myosin light chain kinase, wherein the CaM-binding domain lies next to or partially overlaps with the autoinhibitory domain in the primary sequence (203-205).

Moreover, CaN A1-358 ($\Delta 5$) did not have any change in its maximum activity compared to WT CaN A (Figure 15). However, its basal activity was equivalent to its full

activity level. In other words, the CaN B-dependence of CaN A phosphatase activity was aborted due to the partially removal or disruption of CaN B-binding domain. In a similar way, inactivation of CaM did not change its activity either because of the removal of CaM-binding domain. Thus, CaN A1-358 ($\Delta 5$) was independent of CaN B and CaM (Figure 15). Essentially, CaN A1-358 ($\Delta 5$) was a Ca^{2+} -independent form of CaN. It could be constitutively active because it is unable to sense any Ca^{2+} concentration change. Furthermore, CaN A1-358 ($\Delta 5$) demonstrated the importance of CaN B for CaN A to reach maximal activity. Even when the autoinhibitory domains were removed, CaN A phosphatase activity was at basal level (Figure 15). Similar finding was demonstrated in a previous study (206). Binding of CaN B is required for maximum CaN A activity.

According to the current literature, the CaN A catalytic site is essentially formed by a β -sandwich with a loop, Loop 7, between two β -sheets (207). Loop 7 is very important for enzymatic activity and regulation of CaN (208). It forms close contact with the two immunophilin-immunosuppressant complexes and the autoinhibitory domain (47, 209, 210). Deletion of individual residues in and around Val314 in Loop 7 resulted in reduced sensitivity to CsA but increased sensitivity to FK506; while the inhibition by autoinhibitory domain was removed (208). Moreover, when Asp313, Val314, Tyr315, Asn316 or Lys318 was individually deleted, CaN activity was dramatically increased (211, 212). In contrast, CaN activity was greatly reduced when Leu312 or Asn317 was deleted (211, 212). The CaN B- and CaM-dependence of CaN activity were also removed when Val314 or Tyr315 was deleted (211, 212).

In addition, it has been demonstrated that mutations (Thr352, Leu354, Lys360) in the

CaN B-binding domain result in various extents of resistance to inhibition by CsA and FK506 (213-215). Substitutions of residues 118-125 in CaN B also confer some extent of resistance to the inhibition (213-215).

5.6 Dephosphorylation of ^{32}P -labeled histone

In order to further substantiate the phosphatase activity of WT CaN A and its mutants, we examined dephosphorylation of $[\gamma\text{-}^{32}\text{P}]$ -labelled histone by WT CaN and its mutants since pNPP is not a specific substrate for CaN phosphatase activity. Prior to performing the dephosphorylation of $[\gamma\text{-}^{32}\text{P}]$ -histone, histone was phosphorylated by using cAMP-PK in the presence of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ as described in “Materials and Methods”. $[\gamma\text{-}^{32}\text{P}]$ -labeled histone alone was used as the control representing 100% phosphorylation (Figure 16).

All the experimental groups including WT CaN A and the five mutants were compared to the control. The amount of phosphorylation left in the reaction mixture of each group was calculated as percentages. Two groups of experiments were run separately in the presence/absence of CaM (Figure 16). In the presence of CaM, WT CaN A displayed more than 50% of dephosphorylation activity. CaN A97-511 ($\Delta 1$) dephosphorylated nearly 70% of phosphorylated histone. A similar dephosphorylation ability compared to WT CaN A was observed for CaN A201-511 ($\Delta 2$). However, the remaining three mutants were demonstrated to have stronger dephosphorylation abilities. CaN A1-456 ($\Delta 3$) and CaN A1-406 ($\Delta 4$) removed 90% of phosphate group. CaN A1-358 ($\Delta 5$) removed 75% of phosphate group.

In contrast, when CaM is absent, WT CaN A only dephosphorylated 20% of phosphorylated histone (Figure 16). When there is no CaM, the autoinhibitory domain at the C-terminal region will bind to the active site to inhibit the CaN A phosphatase activity.

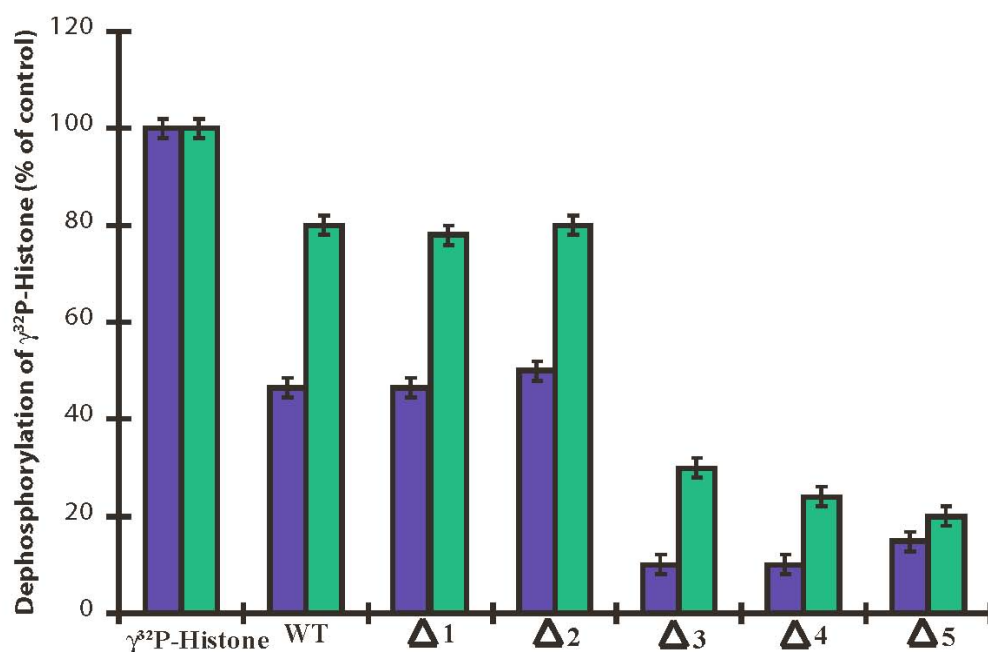


Figure 16. Dephosphorylation of ^{32}P -labeled histone by bovine eye CaN and various truncated mutants. Phosphorylated histone alone; WT, CaN A; $\Delta 1$, CaN A97–511; $\Delta 2$, CaN A201–511; $\Delta 3$, CaN A1–456; $\Delta 4$, CaN A1–406; and $\Delta 5$, CaN A1–358. Phosphorylated histone (0.2 mg/mL) was incubated at 30 °C in a reaction mixture as described in Materials and Methods Section. The eye CaN A or various truncated mutants and CaN B were incubated with 1 mM Ni^{2+} at room temperature for 1 h prior to the incubation of dephosphorylation reaction. The reaction mixture was incubated with (■) and without (■) CaM at 30 °C for 30 min. The data presented are representative of at least three independent experiments.

CaM is needed to bind to CaN A and cause conformational change so that the autoinhibitory domain is positioned away from the active site. Thereby, CaN A phosphatase activity was reduced more than 50% in the absence of CaM. CaN A97-511 ($\Delta 1$) and CaN A201-511 ($\Delta 2$) displayed similar dephosphorylation abilities compared to the WT CaN A. This was expected since both mutants were constructed by progressively deleting the N-terminal region while the autoinhibitory domain is located at C-terminal region. The other three mutants showed slightly reduced dephosphorylation activity in the absence of CaM; although the percentages of phosphate group being removed were still greater than that of WT CaN A (Figure 16). The presence or absence of CaM did not have very much influence on these mutants' phosphatase activity. It is highly possible that the autoinhibitory domain of these three mutants has been removed or disrupted. In addition, the phosphatase activities of CaN A1-358 ($\Delta 5$) in the presence/absence of CaM were very close. It indicates that CaN A1-358 ($\Delta 5$) is CaM-independent.

5.7 Discussion

Ca^{2+} acts as a second messenger in a number of different signaling pathways and plays a critical role in a myriad of different physiological and pathological processes (216, 217). Increase in intracellular Ca^{2+} concentration by stimulating the entry of extracellular Ca^{2+} or by release of Ca^{2+} ions from intracellular stores leads to the binding of Ca^{2+} to CaM, which then converts CaM to an active conformation. This active CaM involves a Ca^{2+} -dependent reversible association of CaM with target protein(s) resulting in a change in target protein activity (218, 219). CaM-dependent enzymes participate in various physiological processes either directly, or indirectly, through the regulation of the activity of other cellular proteins by phosphorylation or dephosphorylation (220). These enzymes are involved in cell proliferation and differentiation by participating in Ca^{2+} -dependent induction of gene transcription (221).

The response of retinal photoreceptor cells to light is a second potential pathway for regulation by CaN. During the photoreceptor cycle a series of reactions result in a net decrease of cGMP concentrations in photoreceptors, causing an inactivation of cGMP-gated cation channels that allows the photoreceptor to extrude ions and the return to a resting potential (185). One of the ions regulated in this process is Ca^{2+} . Intracellular Ca^{2+} concentrations in photoreceptors are also mediated by release from intracellular stores by the interaction of IP3 (186, 187, 222) with its receptor. CaN has been shown to regulate IP3 receptors in other tissues by dephosphorylation (80) and leads us to hypothesize that Ca^{2+} concentrations in

photoreceptors could also be regulated by CaN. These pathways involving CaN could suppress the amplitude of Ca^{2+} oscillations in photoreceptors facilitating a more rapid transition from the light- to the dark-adapted photoreceptor. Such a possible pathway could account for the relatively high amounts of CaN demonstrated in the retina. CaN is also involved in neuronal signal transmission and in the production and maintenance of myelin sheaths (223, 224). The relatively low levels of CaN in eye may make it particularly susceptible to the side effects of the CaN inhibitors Cs A and FK506.

In order to study retina CaN, we have cloned and characterized the bovine retina CaN A and B subunits. Our present study revealed that when compared to bovine brain CaN A, retina CaN A is missing 10 amino acid residues (A-T-V-E-A-I-E-A-D-E). We also observed that the retina CaN A is similar to cardiac CaN A (198). We assessed the role of the different domains of the CaN A subunit in the regulation of CaN activity using WT and various truncation mutants by measuring the Ca^{2+} /CaM-dependent phosphatase activity towards a pNPP substrate. This deletion analysis confirmed that removal of the autoinhibitory domain (residues 457-511) of CaN A resulted in increased CaN A activity. Of particular interest, we find that deletion of the residues N-terminal to the autoinhibitory domain (residues 407-456) also significantly increased CaN A activity. This suggests that CaN A may contain two autoinhibitory domains or that the previously defined autoinhibitory domain extends further towards N-terminal to what had previously been reported (108).

A dinuclear metal center has been proposed to be located in the catalytic site

of CaN A (225, 226). Several studies have identified a “phosphoesterase motif” that is conserved in PP1, PP2A, CaN and many other enzymes involved in the cleavage of phosphoester bonds (227-229). There are several ways that the metal ions of the dinuclear center could function to catalyze the phosphate ester hydrolysis. For instance, the Lewis acidity of the metal ions might act to activate a solvent molecule, such as water, to increase the nucleophilicity of the solvent (230). In addition, metal coordination of the phosphate ester might have positive effects to accelerate hydrolysis (207). For example, the electrophilicity of the leaving group could be increased by neutralizing the negative charge on the oxygen atoms of the phosphate ester (207). The metal ions could also help to stabilize the charge formation on the leaving group (207).

In addition to the endogenous dinuclear metal ions, CaN activity is also regulated by exogenous divalent metal ions (66). Generally, these metal ions are classified into five categories based on their effects on CaN activity (66). First, the transition metal ions include Ni^{2+} , Mn^{2+} and Co^{2+} are the strongest stimulators of CaN activity (192, 231-236). Mg^{2+} falls into a category of its own because of several unique features of its activation (234, 235). The third category includes Ca^{2+} , Ba^{2+} and Sr^{2+} , which are relatively less effective in activating CaN (232, 234, 235). The fourth category includes Be^{2+} , Cu^{2+} and Fe^{2+} , which do not stimulate CaN activity (192, 231). The fifth category includes Zn^{2+} and Cd^{2+} , which inhibit CaN activity (37, 237). Interestingly, bovine retina CaN was found to have a different feature from the previously reported. Fe^{2+} was found to stimulate retinal CaN activity significantly. It

is highly possible that the interaction between Fe^{2+} and bovine retina CaN is different from the interaction between Fe^{2+} and CaN of other sources. However, the exact reason of such stimulation effect is yet to be explored.

In addition, it has been proposed that metal ion stimulators might regulate the substrate specificity of CaN (66). For example, when myelin basic protein is used as substrate, CaN is stimulated by Mn^{2+} but inhibited by Ni^{2+} or Co^{2+} (233). Dephosphorylation of histone H1 and phosphorylase α by CaN were stimulated by Ni^{2+} to different extents (231). CaN activity were stimulated eight- and five-fold, respectively. This suggests that the stimulation effect is also dependent on the nature of phosphorylation site in substrate (66).

Role of Ca^{2+} in CaN activity is closely related to the two protein regulators: CaN B and CaM, of CaN A. Many reports have revealed that Ca^{2+} exerts little or no stimulation on CaN activity in the absence of CaM (66). Ca^{2+} can not substitute for transition metal ions or Mg^{2+} to fulfill the metal ion requirement of CaN activity (237). Ca^{2+} is required for activation of CaM as well as the association of CaM and CaN B with CaN A. Both CaM and CaN B have four Ca^{2+} binding sites. Although CaN B and CaM share sequence homology, they are not replaceable for each other in order to activate CaN A. Both CaM and CaN B are required to fully activate CaN A phosphatase activity.

The interaction between CaN and metal ions occurs at multiple levels. Firstly, CaN B is a Ca^{2+} -binding protein. It enables the association of CaN A and CaN B upon binding of Ca^{2+} to CaN B (23). Secondly, Ca^{2+} is required for CaM to further activate

CaN A phosphatase activity (24). Thirdly, several divalent metal ions activate the enzyme to different extent (67, 236). Therefore, CaN contain endogenous metal ions; also, it interacts with exogenous metal ions and CaM in a metal ion-dependent interaction.

Calpains are Ca^{2+} -activated cysteine proteases which are major mediators for Ca^{2+} signals in many biological systems including the altering the function of kinases and phosphatases (238, 239). There are at least two types of calpains, μ - and *m*-calpains, which require micromolar and millimolar concentrations of Ca^{2+} for activation, respectively (238). Ca^{2+} -dependent cysteine proteases, such as calpain have suggested to cleave polypeptide sequences enriched in proline (P), glutamic acid (E), serine (S) and threonine (T), known as PEST motifs (240). Calpastatin, an endogenous protein inhibitor regulates the activity of calpains (241). Deregulation of the calpain-calpastatin system has been implicated in various neurodegenerative disorders (242, 243). PEST sequences are believed to be the intramolecular signals for rapid proteolytic degradation by *m*-calpains. It has been suggested that PEST regions may not be responsible for the calpain-mediated proteolysis (244).

It has been reported that bovine brain 60 kDa CaMPDE has a single strong PEST motif (amino acid residues 73-94), and a PEST score 7.36 (245). It has been reported that PDE1A2 isozyme is proteolysed by calpain into a 45 kDa catalytic fragment and a 15 kDa fragment. The cleavage occurs between residues ¹²⁶Gln and ¹²⁷Ala (246). This eliminates the CaM-dependent activity of carboxy termini of 60 kDa CaMPDE isozyme, resulting in its conversion to a totally CaM-independent form

(246).

The domain organization of CaN A is shown in figure 3. CaN A contains catalytic (residues 70-333) and regulatory (residues 390-521) domains. The regulatory domain contains a CaN B binding helix, a CaM-binding domain and an autoinhibitory domain. The regulatory domain is readily susceptible to proteolysis in the absence of CaM (247). We reported that the *m*-calpain-mediated proteolysis of CaN A resulted in the induction of CaN phosphatase activity (108) and CaN A became completely CaM-independent. Wang *et al.* (248) reported that, upon proteolysis of CaN by μ -calpain, the phosphatase activity measured in the presence of CaM increased by 300%. In addition, CaN activity increased by 50% in response to *m*-calpain proteolysis (measured in the presence of CaM). Tallant *et al.* (249) reported that CaN was converted to an active CaM-independent form by μ -calpain-mediated proteolysis. Trypsin proteolysis of CaN in the absence of CaM resulted in the degradation of the 60 kDa subunit to a 43-45 kDa fragments that became CaM-independent after proteolysis (250).

We observed CaN A was proteolysed to a 46 kDa fragment in the presence of Ca^{2+} and 48 and 54 kDa in the presence Ca^{2+} /CaM (108). All three of these proteolytic products possessed phosphatase activity, suggesting that each contains the N-terminal catalytic domain of CaN A. The CaM overlay experiment showed that the CaM-binding domain was present in the 54 kDa proteolytic fragment suggesting the *m*-calpain cleavage occurs after the CaM-binding domain of CaN A. However, 46 and 48 kDa fragments lacked the CaM-binding domain, suggesting they were cleaved N-

terminal to the CaM-binding domain. However, further studies are warranted to identify precise cleavage site(s) by *m*-calpain on CaN A.

A series of truncated mutants were constructed, expressed and purified as described in Materials and Methods. Figure 13 shows a schematic representation of various deletion mutants for CaN A. Two different mutants were prepared by deleting progressively larger N-terminal regions of catalytic domain of CaN A to define which region(s) of this domain were essential for CaN phosphatase activity. **Δ1** CaN A97-511 and **Δ2** CaN A201-511 mutants were constructed by deleting the indicated number of amino acid residues. No significant change in phosphatase activity was observed for mutants **Δ1** CaN A97-511 compared to WT CaN A, while **Δ2** CaN A201-511 showed 20% decrease in CaM-dependent phosphatase activity (Figure 15). To further substantiate the CaN phosphatase activity measured using pNPP as a substrate, the dephosphorylation of [γ - ^{32} P]-labeled histone as a substrate was carried out. We observed a similar pattern of dephosphorylation of histone using WT CaN and various truncated mutants (Figure 16). These results indicated that the deletion of the first 97 amino acids of CaN A had no impact on the phosphatase activity of retinal CaN.

Three C-terminally truncated mutants were constructed to assess the role of different regions with the regulatory domain of CaN A in the regulation of CaN A phosphatase activity. Furthermore, the truncated mutant **Δ3** CaN A1-456 was prepared by deleting ten additional residues within the autoinhibitory domain of CaN A. The retinal CaN phosphatase activity was increased to 31% in the mutant **Δ3** CaN A1-456.

Moreover, we observed CaM was essential for the maximum activation of **Δ3** CaN A1-456 mutant, demonstrating that the autoinhibitory domain alone is not responsible for the inhibition of phosphatase activity of CaN. These results are in agreement with the report of Tokoyoda *et al* (200) and Lakshmikuttyamma *et al* (108). Furthermore, we have generated a further truncated CaN A mutant (**Δ4** CaN A1-406), to evaluate which part of the CaM-binding domain was responsible for CaM binding. This further deletion results in further phosphatase activation. For this particular mutant no significant change was observed in CaM-independent and dependent phosphatase activity of CaN, and it showed a 46% higher activity compared to WT CaN. This result revealed that all amino acid residues (391-414) in the CaM-binding domain are not involved in the regulation of phosphatase activity. From these data it appears that N-terminal sequences of CaM-binding domain (407-414) are responsible for the inhibition of phosphatase activity of retinal CaN. The truncated mutant **Δ5** CaN A1-358 was made by deletion of amino acid residues EKVTEMLVDV of the CaN B binding domain. Compared to WT CaN A, no change in phosphatase activity was observed for **Δ5** CaN A1-358 by the addition of CaN B. Previous reports demonstrated that the peptide corresponding to the residues 348-367 in brain CaN A was able to inhibit the phosphatase activity and indicate the participation of these residue in the binding of CaN B to the catalytic subunit [251].

Deletion of the autoinhibitory domain and CaM-binding domain has been shown to be essential for the induction of CaN activation (200). The analysis also indicated that the deletion of only one domain was insufficient for the activation of

CaN. The synergy between these two domains is crucial for the Ca^{2+} dependent regulation of phosphatase activity of CaN (200). The removal of these two domains during calpain-mediated proteolysis may be the reason for the enhanced activity of proteolysed CaN. With most CaM regulated enzymes, the mechanism of CaM activation is believed to involve binding to the CaM-binding domain, and consequent displacement of an auto inhibitory domain. This model was supported by crystal structure analysis of the native CaN molecule showing that the structure of the CaM-binding domain to the COOH terminal end was not visible (47, 209). Thus it is likely that the COOH terminal end from the CaM-binding domain may be structurally unstable.

In recent years, it has been increasingly recognized that Ca^{2+} /CaM-dependent protein phosphatase CaN provides a critical link between Ca^{2+} regulation, synaptic plasticity, and cell survival (251). Over-expression of CaN in young adult animals leads to altered synaptic function and memory retention deficits (252). In hippocampal neurons, activation of CaN results in the inhibition of the release of neurotransmitters, glutamate and γ -aminobutyric acid (253), as well as desensitization of post synaptic NMDA receptor-coupled Ca^{2+} channels (254). Excess stimulation of CaN, by raising intracellular Ca^{2+} levels, releases CaN into the cytosol. In the cytosol, it may cause neuronal apoptosis via dephosphorylation of key cytosolic components, such as the Bcl family member Bcl-2 associated death protein (BAD) and cAMP response element binding protein (CREB) (253). The altered regulation of CaN is one of the processes that could link Ca^{2+} dyshomeostasis to age related changes in

neuronal function and cognition (255). CaN inhibitors FK 506 and Cs A are used for differential neuroprotection following ischemia and treatment of ameliorated glutamate toxicity and delayed neuronal death (255-257). Recent studies suggest that a significant increase in both basal and maximal activity of CaN were observed in epileptic rats (258). These results support the hypothesis that CaN is toxic for neurons and may contribute to various neurological disorders. The activation of CaN by calpain mediated proteolysis could be a regulating mechanism for the over-expression of this enzyme in brain neurons. Alterations in intracellular Ca^{2+} homeostasis and the activation of calpains have been implicated in various neurodegenerative disorders like Alzheimer's disease, ischemia and epilepsy (242, 259-261).

The major signaling pathway of CaN in the development of malignancy may be through NFAT. In T cells, CaN-NFAT signaling represents a critical event for mediating cellular activation and the immune response (262, 263). Cell-mediated immune responses are an essential aspect of tumor-host interactions in colorectal cancers. The progression from precancerous (adenomatous) colon polyps to malignant colorectal cancer depends on a complex pathway involving the activities of activated T lymphocytes (264). The important role of CaN in T-cell activation is underscored by the effects of immunosuppressive drugs, such as CsA and FK506, which inhibit CaN activity (265). The activation of T cell receptors recruit a large array of necessary intracellular signaling mediators such as mitogen-activated protein kinase family members, tyrosine kinases (Fyn-T, Lck, Syk, and Zap70) NF-kB, Jak/Stat, PKC θ and various cytokines (266-270). The elevated levels of IL-8 gene expression in human

colonic epithelial cells occur through the activation of nuclear factor-kappa B (NF- κ B) (271) and are regulated by CaM-dependent CaN (272). Elevated levels of circulating plasma cytokines IL-6 and IL-8 have been reported in cancer patients (273). RelA/NF- κ B, an important inhibitor of apoptosis is significantly increased in the transition from adenoma with low dysplasia to adenocarcinoma in colorectal tumorigenesis (274). It has been reported that CaN may regulate integrin-dependent cell adhesion and spread of Colo201 cells (275). Furthermore, CaN may also be involved in the regulation and establishment of new adhesive interactions in HT29, a colon cancer cell line (276).

The involvement of CaN with various apoptotic factors and whether this Ca²⁺/CaM-dependent phosphatase has any role in inhibiting apoptosis is unclear. Simizu et al. reported that Bcl-2 inhibits CaN-mediated Fas ligand expression in anti-tumor drug-treated baby hamster kidney cells (277). Recently Ca²⁺-dependent interaction of CaN with Bcl-2 was reported in neuronal tissues (278). CaN bound to Bcl-2 is an active phosphatase but is unable to promote the nuclear translocation of NFAT, a transcription-factor required for induction of interleukin-2 expression, suggesting a mechanism by which Bcl-2 suppresses NFAT activity. Direct interaction between Bcl-2 and CaN has been reported in BHK-21 cells transfected with both proteins (279). Dephosphorylation of Ser70 of Bcl-2 by CaN is required for antiapoptotic effects of Bcl-2. The overexpressed CaN in human colorectal adenocarcinoma may provide protection against apoptosis by dephosphorylating Bcl-

2 (280). In apoptosis, proteolysis is very important in altering or disabling the function of many enzymes involved in signal transduction.

6.0 CONCLUSION

Both CaN A and CaN B recombinant proteins were successfully cloned and purified from bovine retina in this study. The DNA sequence of CaN A was obtained and deduced to amino acid sequence. The peptide sequence of bovine retina CaN A was 10 amino acids shorter than that of bovine brain CaN A (Figure 8). There was one amino acid substitution at 367. Highly conserved CaN A sequence in mammalian tissues implies important physiological roles of CaN. Reconstitution assay was carried out to confirm that CaN phosphatase activity achieves maximal level at 1:2 molar ratio of CaN A/CaN B (Figure 11). It was also found that Ni^{2+} is the strongest stimulator of CaN phosphatase activity while Zn^{2+} inhibits the activity (Figure 12). However, Fe^{2+} also stimulated CaN phosphatase activity. Deletion analysis was carried out to study the domain organizations of CaN A. It indicates that the amino acid residues 97-201 are essential to the phosphatase activity because CaN A201-511 showed reduced activity level (Figure 14). It appears that there might be another inhibitory site presents between amino acid residues 457-414 because CaN A1-406 showed increased activity level compared to CaN A1-456. As a result, CaN A from bovine retina possesses some distinct structural features compared to bovine brain CaN A. The unique structural features might result in some different catalytic and regulatory characteristics.

In order to further study this protein, the three-dimensional structure of bovine retina CaN A needs to be resolved. The understanding of unique structural and

biochemical characteristics of bovine retina CaN A would provide clues to investigate the physiological roles of such protein in eye. The response of retinal photoreceptor cells to light might be a potential pathway for CaN to be involved. During photoreceptor cycle, a series of biochemical reactions including inactivation of rhodopsin through phosphorylation and decrease of cGMP concentration through action of PDE will take place. CaN phosphatase activity could act on both events to bring photoreceptor cells to a state ready for next cycle of response to light. Such a possible pathway could account for the relatively high level of CaN phosphatase activity in retina (180). The present study demonstrates the first investigation on the structural and biochemical characteristics of CaN from bovine retina. It also provides some fundamental knowledge of retina CaN for further studies on eye diseases.

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