Localization and Characterization of Calcineurin in Bovine Eye

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PURPOSE. There are several aspects of the visual system that may be regulated by Ca 2+ - and calmodulin (CaM)-stimulated protein phosphatase. In the present study, the distribution and characterization of calcineurin (CaN) in bovine eye was determined.

METHODS. Whole bovine eyes were either homogenized for purification or regionally dissected to determine CaN localization and activity. Dissected tissues were homogenized and Western blot analysis performed, using polyclonal anti-CaN antibodies, and assayed using p-nitrophenyl phosphate (PNPP) as a substrate to determine the dephosphorylation activity of CaN. Fresh samples were then prepared for immunohistochemistry and probed with polyclonal anti-CaN antibodies.

RESULTS. CaN was found to be present in all eye tissues, although activity and protein expression varied. The highest levels of CaN activity and protein expression were found in the optic nerve, retina, and cornea. Immunohistochemical methods displayed similar results with additional staining of the optic nerve vasculature. Assays of purified CaN demonstrated that bovine eye CaN had regulatory properties similar to CaN isolated from other tissues. Probing eye tissues with CaN A isoform-specific antibodies demonstrated that eye tissues displayed variable distributions of the CaN α and CaN β isoforms.

CONCLUSIONS. The presence of CaN in the bovine eye provides a physiological pathway by which the phosphorylated state of proteins and intracellular Ca 2+ concentrations can be coordinated. The authors propose that CaN is involved in the immunologic privilege of the cornea, retinal signal transmission, and the toxic effects of immunosuppressants on the eye. Further in vivo studies of CaN function are necessary to understand the contributions of CaN to ocular physiology. (Invest Ophthalmol Vis Sci. 2002;43:15–21)

Calcineurin (CaN) is an 80-kDa protein phosphatase that is regulated by intracellular Ca 2+ concentrations and is capable of regulating a variety of protein substrates by dephosphorylation. The inositol 1,4,5-trisphosphate (IP3) receptor1 is a cytosolic protein that is inactive in its phosphorylated state. The activation of T cells by receptor stimulation causes influx of cytoplasmic Ca 2+ into the cell. CaN is then fully activated, dephosphorylating NF-AT, thus allowing it to enter the nucleus and promote gene transcription.19,20 The activity of CaN can be further stimulated by divalent metal cations such as Ni 2+ and Mn 2+ in vitro.16

The most clinically important feature of CaN is its selective inhibition by the immunosuppressants cyclosporine and FK506 (tacrolimus).17 Inhibition of CaN by immunosuppressants was first observed in T cells, and it was later discovered that many side effects of immunosuppressants on body organs can be attributed to inhibition of CaN in those tissues.18 The central protein regulated by CaN in immunosuppression is the nuclear factor of activated transcription (NF-AT). NF-AT is a cytosolic protein that is inactive in its phosphorylated state. The activation of T cells by receptor stimulation causes influx of Ca 2+ into the cell. CaN is then fully activated, dephosphorylating NF-AT, thus allowing it to enter the nucleus and promote gene transcription.19,20 The dephosphorylated intranuclear NF-AT then promotes the expression of cytokines necessary for a rapid and effective T-cell response.20 It is the inhibition of CaN in this process that mediates the pharmacologic response seen in immunosuppression. This suppression of immune function has found clinical use in organ transplantation and in the treatment of certain inflammatory diseases.21–23

Originally discovered and purified as an inhibitor of CaM-dependent phosphodiesterase in bovine brain,24–25 CaN has since been characterized in numerous other tissues including kidney, liver, muscle, and T lymphocytes.16,18,20 Previous examination of CaN in the eye demonstrated its presence in chick retina26 and more recently in the developing and mature mammalian retina.27 To date, however, a comprehensive study of CaN throughout all tissues of the visual axis has not been undertaken, thus forming the rationale for the present study. We were able to identify and purify CaN from whole bovine eye extracts and also described the distribution of CaN by Western blot analysis and immunohistochemistry. By examin-
ing the localization, regulatory properties, and activity of CaN in all portions of the eye, we hoped to gain insight into the physiological roles CaN may play in maintaining mammalian ocular function or its contribution to disease states of the eye.

METHODS

Materials

All methods conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Visual Research. Fresh bovine eyes were obtained from an abattoir, transported on ice, and either used immediately or stored at −70°C. CaM was purified from bovine brain as described previously. 4 General laboratory reagents were obtained from Sigma Chemical Co. (Toronto, Ontario, Canada) and BDH Inc. (Toronto, Ontario, Canada) and were of analytical grade.

Preparation of Crude Homogenates

Selected bovine eye tissues (cornea, lens, iris, ciliary body, vitreous body, retina, choroid, sclera, and optic nerve) were dissected and homogenized, using a homogenizing probe for 5 minutes in 2 mL ice-cold 20 mM Tris-HCl buffer (pH 7.5), per gram of tissue. Crude homogenates for purification were prepared from whole bovine eyes using a meat grinder followed by homogenization in a blender for 1 minute in the previously mentioned buffer containing 2 mM EDTA, 100 mg/mL phenylmethylsulfonyl fluoride, 100 mg soybean trypsin inhibitor, and 200 mg/L benzamidine. These protease inhibitors were included in the buffers up to the CaM-Sepharose-4B affinity chromatography step. The homogenates were centrifuged at 10,000g for 25 minutes, and the supernatant was filtered through glass wool.

2-Mercaptoethanol and EGTA were added to the supernatants of samples to final concentrations of 10 and 0.1 mM, respectively.

Phosphatase Assay

CaN phosphatase activity was assayed using p-nitrophenyl phosphate (PNPP, Sigma Chemical Co.) as a substrate. 28 Twenty-five micrograms of crude tissue homogenate and buffer were added to the assay mixture to a total volume of 1 mL. One unit of phosphatase activity was defined as the amount of dephosphorylation resulting in an optical density of 0.1 at 30°C after 30 minutes’ incubation. All assays were performed in duplicate in three separate samples.

Production of Polyclonal Antibody against CaN

Antibodies were raised in New Zealand White rabbits given multiple subcutaneous injections of 50 μg bovine brain CaN emulsified with complete Freund’s adjuvant (CFA) at multiple sites. The first blood samples were obtained on day 36, and two boosters were given with the same 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 ELISA, as described earlier. 29 The antibody was purified through a protein A-Sepharose-4B column, pre-equilibrated with 100 mM Tris-HCl (pH 8.0), followed by elution of bound IgG with 100 mM glycine (pH 2.5). Immediately after elution, the pH was adjusted to 8.0 with 1 M Tris, and the immunoglobulin was dialyzed against phosphate-buffered saline (PBS) overnight. The antibodies were demonstrated to be specific for CaN (data not shown). The dialyzed sample was stored at −70°C in small aliquots containing 0.9 mg of protein per milliliter.

SDS-PAGE (10%) was performed according to the method of Laemmli. 30 CaN A isoform-specific antibodies (Aa and AB) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Western blot analyses were performed essentially as described by Towbin. 31 Samples were subjected to 10% SDS-PAGE, transferred to nitrocellulose membranes (Bio-Rad Laboratories Ltd., Mississauga, Ontario, Canada), and blocked with 5% milk powder in PBS with Tween 20 (PBST) for 1 hour at room temperature. Polyclonal and isoform-specific antibodies were diluted (1:1000) in blocking buffer and incubated with the membrane overnight at 4°C. Membranes were then washed once with PBST for 30 minutes and washed three times for 5 minutes each to remove unbound antibody. Horseradish peroxidase-conjugated secondary antibodies (Bio-Rad Laboratories Ltd.) were diluted (1:2000) and incubated with membranes for 1 hour at room temperature. Membranes were then washed once with PBST for 1 hour and washed twice for 5 minutes each to remove unbound secondary antibody. Immunoreactive bands were visualized on imaging film (Eastman Kodak Co., Rochester, NY) using chemiluminescence reagents (NEL Life Sciences Products, Inc., Boston, MA). Quantitative analysis of Western blot analysis was performed using imaging software obtained from the National Institutes of Health (http://www.ncbi.nlm.nih.gov/nlh-image/download.html).

Immunohistology

Immunohistology was performed as described previously. 32 Bovine eyes, fixed in 10% formaldehyde and dehydrated in ascending solutions of ethanol and xylene, were embedded in paraffin. Five sections of 7-μm thickness were prepared from tissue blocks and placed on slides coated with silane. The slides were kept at 55°C for 45 minutes in an oven to improve adherence of sections.

The sections were deparaffinized and rehydrated in xylene and descending concentrations of ethanol. Endogenous peroxidase was neutralized by a 30-minute incubation in 0.5% hydrogen peroxide in methanol. The antigens were unmasked by treating the sections with 2 mg pepsin per milliliter 0.01 N HCl for 45 minutes. This incubation time was determined with a series of trials and was found to yield maximum staining.

The sections were then blocked with 1% bovine serum albumin in PBS for 30 minutes, followed by incubation with anti-CaN polyclonal antibody (1:100) for 90 minutes and with HRP-conjugated secondary antibodies (1:100–1:400; Dako Corp., Carpinteria, CA) for 45 minutes. The reactions were color developed with a peroxidase substrate kit (SK4600; Vector Laboratories, Burlingame, CA). Control experiments included omission of either the primary antibody or both primary and secondary antibodies to determine, respectively, nonspecific binding of the secondary antibody and inhibition of endogenous tissue peroxidase. Some of the sections were stained with von Willebrand factor antibody (vWF; 1:200; Dako Corp., Carpinteria, CA) for 45 minutes. The sections were counterstained for 2 to 4 minutes with methyl green. The slides were examined and images captured on an image analysis system (Northern Eclipse, Empix Imaging, Mississauga, Ontario).

Other Methods

Protein concentrations were determined by the method of Bradford using bovine serum albumin as a standard. 33

RESULTS

CaN Activity in Eye Tissues

Crude homogenates of cornea, lens, iris, ciliary body, vitreous, retina, choroid, sclera, and optic nerve were assayed for phosphatase activity using PNPP, either in the presence of Ni2+ and CaM or EGTA. The presence of Ni2+ and CaM resulted in higher phosphatase activity in all tissues compared with that in Ca2+-free samples (i.e., in the presence of EGTA). The highest levels of CaN activity were found to be in the optic nerve, retina, and cornea, which displayed 18.9, 17.2, and 16.6 U of activity per milligram of tissue, respectively (Fig. 1). Lenses contained very little activity (0.15), whereas all other tissues contained activities between 4.5 and 11.1 U/mg of tissue.
Western Blot Analysis of Eye Tissues

Crude tissue homogenates were probed with polyclonal antibodies raised against bovine brain CaN. Western blot analysis of eye samples revealed immunoreactive bands in all eye tissues (Fig. 2A). These bands were 60- and 19-kDa, corresponding to the A and B subunits of CaN, respectively. Corneal samples displayed strong immunoreactivity for CaN A subunit with weak to absent immunoreactivity for CaN B subunit. Lens tissue displayed the inverse of this phenomenon with large amount of CaN B subunit visualized but no apparent CaN A subunit. The amount of CaN protein expression correlated quite well with the activity data in Figure 1, with the exception of the optic nerve. The CaN A subunit in cornea also displayed an apparent molecular weight that was on average higher than 60 kDa. All other tissues displayed more consistent ratios of CaN A and CaN B expression. Quantitative analysis of Western blot analysis for bovine eye tissue was performed using a known quantity of bovine brain CaN as a standard on the same blot (lane containing standard not shown). CaN A expression ranged from 12.8 (cornea) to 0.27 (lens) mg CaN per kilogram of tissue, whereas CaN B expression was much lower, varying between 0.32 (cornea) to 5.17 (lens) mg/kg of tissue (Figs. 2B, 2C).

Immunohistochemistry

Sections from corneas (Fig. 3A), retina (Fig. 3C), and optic nerve (Fig. 3E) incubated with only secondary antibody showed no staining indicative of absence of nonspecific binding, whereas those incubated with anti-vWF antibody outlined the vasculature (data not shown). An adjacent section of cornea exposed to anti-CaN antibody revealed intense staining in the epithelium, but weak reaction in the substantia propria (Fig. 3B). The antibody showed similar staining in retina but only weak staining in the choroid (Fig. 3D). The optic nerve and its blood vessels stained positively with the antibody (Fig. 3F). A high-magnification view of the retina (Fig. 3G) depicts intense staining in various cell layers.

Purification of CaN from Whole-Eye Tissue

Crude homogenates of whole bovine eye (500 g) were prepared as described in the Methods section. CaN was purified to apparent homogeneity using diethylaminoethyl (DEAE)-Sepharose CL-6B, Affi-Gel Blue (Bio-Rad Laboratories Ltd.), and CaM-Sepharose 4B–affinity chromatography according to the procedure of Sharma et al. Results not shown. Total CaN activity from the soluble fraction of bovine eye was determined to be 1155 U. Column chromatographic techniques were effective in purifying CaN to homogeneity. The specific activity of the purified samples ranged from 58 to 73 U/mg per minute. Purified CaN was used for inhibitory, regulatory, and immunologic studies.

Activation of CaN by Divalent Metal Cations and Inhibition by CaM Antagonists

Purified CaN was assayed with Ni$^{2+}$ and CaM to determine maximum in vitro activity. In the presence of Ni$^{2+}$ alone, CaN activity was 38%, compared with the activity observed in the presence of Ni$^{2+}$ and CaM. CaN also displayed activation by other divalent cations such as Mn$^{2+}$ and Ca$^{2+}$. The antipsychotic drug fluphenazine has been shown to be a reversible inhibitor of CaM, and its addition to reaction assays reduced CaN activity to 57%, compared with similar reactions without fluphenazine. In the absence of Ca$^{2+}$, CaM, or metal cations, the endogenous activity of CaN was found to be 5% of maximally activated levels.
Determination of the CaN A Isoform

It has been shown that CaM-regulated enzymes exist in multiple isoforms with unique functions and characteristics, and CaN A also has been demonstrated to exist in isoforms. Because the eye is a heterogeneous organ containing many different tissues, immunoblot analysis of CaN was performed in different regions of the eye. The expression of the two major isoforms of CaN A, CaN Aα and CaN Aβ, were examined by probing eye tissues with isoform-specific polyclonal antibodies. CaN Aα and CaN Aβ antibodies reacted with all eye samples examined with the exception of samples from lens and cornea (Fig. 4). Lens tissue displayed no detectable reaction for either of the CaN A isoforms, an observation also apparent in Figure 2. Cornea however displayed distinctive isoform expression. Strong reaction was observed for the CaN Aα isoform in corneal samples (Fig. 4, top), whereas there was negligible expression of the CaN Aβ isoform (Fig. 4, bottom).

FIGURE 3. (A, C, E) Sections of cornea, retina, and optic nerve, respectively, that were exposed only to secondary antibody and, therefore, show no staining. (B) Intense reaction for CaN in the corneal epithelium (double arrows) but weak in the substantia propria (S). (D, F) CaN was localized in retina (R) and optic nerve (Op) including the blood vessels (single arrow) but was weak in the choroid (Ch). (G) High-magnification view of retina, showing intense reaction for CaN in various cell layers. Magnification, (A-F) ×200; (G) ×800.

FIGURE 4. Western blot of bovine eye tissues with CaN A isoform-specific antibodies. Thirty-five milligrams of protein from various eye tissues was blotted with polyclonal antibodies raised against CaN Aα or CaN Aβ isoforms.
DISCUSSION

CaN was identified and characterized in bovine eye in the present study. All eye tissues contained activity, and protein expression was determined by Western blot analysis and immunohistochemistry. CaN A isoform-specific antibodies indicated that eye tissues express both CaN Aα and CaN Aβ, although both isoforms are not present in all eye tissues. Purified bovine eye CaN displayed similar regulatory properties as observed in CaN from other tissues. CaN in bovine eye displayed stimulation by Ca2+, CaM, and divalent cations.

Western blot analysis indicated that both subunits of CaN are expressed in eye, and antibodies specific for two isoforms of CaN A are cross-reacted with various eye tissue samples. The implications for the expression of CaN A isoforms stems from the distribution of these isoforms in other tissues. The CaN Aβ isoform is predominantly expressed in cells of lymphoid lineage and is believed to be the isoform responsible for mediation of the immune response. The CaN Aα isoform is more widely distributed, and its inhibition is believed to cause many of the detrimental side effects of immunosuppression. The presence of both isoforms of CaN A in eye suggests CaN may be involved in immunoregulation and other presently unknown functions (Fig. 4). However, the expression of only the CaN Aα isoform in cornea is unique (Fig. 4, top). The amount of CaN determined by quantitative Western blot analysis (Fig. 2) in the eye further demonstrates that CaN is present in relatively low amounts, compared with levels in other tissues.

The recent study by Nakazawa et al., demonstrated CaN distribution in developing rat and mature rat, bovine, and human retina, and further demonstrated that distribution of CaN was limited to the cell bodies of ganglion cells and cells of the inner nuclear layer. Our study further confirms this distribution; however, when probing with isoform and subunit-nonspecific antibody we found the distribution of CaN to be more widespread within retinal tissue cell types (Fig. 3G). Our observations agree with the study by Nakazawa et al., in that we were able to demonstrate expression of both the CaN Aα and CaN Aβ isoforms in the retina (Fig. 4).

The cornea is a unique tissue in many respects, the most noteworthy of which is its immune privilege, which allows for the high success rate of unmatched corneal transplantation. A recently discovered factor involved in corneal immune privilege is the constitutive expression of Fas ligand (Fas L) by corneal epithelial and endothelial cells. FasL, a transmembrane protein that is a member of the tumor necrosis factor family, is limited to activated T cells and to certain cells in the testes and cornea. The receptor for FasL (Fas”) conversely is more widely expressed, being found on the surface of many cells, including T-cells. The interaction between FasL and its receptor results in the forcing of the Fas” cell to undergo apoptosis. The importance of FasL in the survival of corneal grafts is emphasized by experiments in mice with neither Fasl nor Fas”, which demonstrate an increased rate of rejection of corneas taken from FasL-deficient mice and from hosts that do not have the Fas” receptor.

Recent studies have shown CaN activity to be a component that is necessary in the functional expression of Fasl. We have shown in this study that corneal epithelial tissue expresses large quantities of CaN (Figs. 2A, 3B). It was also noted that CaN in cornea had an almost undetectable expression of the CaN B subunit (Fig. 2C). The possibility of corneal CaN’s being a unique isoform, perhaps with higher endogenous activity, is thus speculated and further investigation into this is warranted. The further possibility of the involvement of CaN in both ischemic and apoptotic processes in cornea, both of considerable significance, may also be of importance in future studies.

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. One of the ions regulated in this process is Ca2+. Intracellular Ca2+ concentrations in photoreceptors are also mediated by release from intracellular stores by the interaction of IP3 with its receptor. CaN has been shown to regulate IP3 receptors in other tissues by dephosphorylation and leads us to hypothesize that Ca2+ concentrations in photoreceptors could also be regulated by CaN. These pathways involving CaN could suppress the amplitude of Ca2+ 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 (Fig. 3G).

The immunosuppressants cyclosporine and FK506, both known selective inhibitors of CaN, are very effective in suppressing immune reactions but also have many systemic side effects, including ocular complications. Cyclosporine, which is neurotoxic, can cause cortical blindness and severe retinal disease. We were able to demonstrate expression of CaN in the vasculature of bovine eye (Fig. 3D), thus supporting a possible ischemic mechanism of immunosuppressant toxicity. CaN is also involved in neuronal signal transmission and in the production and maintenance of myelin sheaths. The relatively low levels of CaN in eye may make it particularly susceptible to the side effects of the CaN inhibitors cyclosporine and FK506.

Our studies demonstrate the first characterization and localization of CaN in the entire eye. Expression of CaN in eye, as described in the present study, provides a framework for further studies on immunosuppressants and their ocular effects. Currently, research is being conducted into the development of synthetic corneas for use in corneal transplantation. Ensuring expression of CaN and FasL in these tissues will be an important factor in determining long-term survival of these grafts. Culturing conditions with sufficient Ca2+ concentrations to maintain CaN activity should add to the survival of these corneas. Furthermore, the potential future use of topical immunosuppressants in the treatment of eye disease and the role CaN plays in certain ocular pathologic states should be examined. Studies into the physiological function of CaN in vivo should be undertaken to examine these facets of ocular function.

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