Molecular cloning and characterization of porcine calcineurin-α subunit expression in skeletal muscle

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ABSTRACT: The calmodulin/Ca2+-dependent serine/threonine phosphatase, calcineurin (CaN), has been implicated in controlling muscle fiber phenotype. However, little information is available concerning the expression of CaN in porcine skeletal muscle. Therefore, the porcine CaNα (CaN-A) was cloned by reverse transcription-PCR and its expression characterized in selected porcine skeletal muscles. We successfully cloned porcine CaN gene using semitendinosus muscle (GenBank accession number AF193515). Sequence analysis showed both the full length and a 30-bp deletion splice variant in coding region of the gene reported in other species. The deduced AA sequence showed 99.4% homology with the rat CaN-A δ isoform gene. Real-time PCR analysis showed CaN is present in all tissues. However, using primers targeting the region containing the 30-bp deletion, the full length sequence is only found in skeletal muscle and brain tissues. Using a CaN-A monoclonal antibody, we localized CaN-A in porcine LM and soleus muscle and the red and white portions of the semitendinosus muscle. The CaN-A protein was abundant in fast fibers and primarily localized in the cytoplasm, whereas slow fibers expressed reduced abundance of CaN-A. Further studies are required to understand the functions of CaN-A isoform in skeletal muscle.

Key words: calcineurin, fiber type, gene sequence, pig, protein phosphatase 2B

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INTRODUCTION

Mammalian locomotion depends on the existence of 2 types of skeletal muscle. Red or slow muscles generate a sustained contraction that is paramount for maintaining postural stature, whereas white or fast muscles are involved in powerful force output (Goldspink, 1999). Red muscles are mainly composed of oxidative or type 1 and oxido-glycolytic type 2A fibers, whereas white muscles are principally composed of glycolytic 2X(D) and 2B fibers. Skeletal muscle fiber phenotype is remarkably plastic for adaptation to physical activity, hormonal, and disease status (Buonanno and Rosenthal, 1996).

A relationship between innervation and muscle fiber phenotype regulation has long been known (Buller et al., 1960). Innervation can direct muscle fiber-specific gene expression (Hughes et al., 1999), and those changes are mediated by the metallo-serine-threonine phosphatase calcineurin (CaN; Chin et al., 1998; Dunn et al., 2001), also known as protein phosphatase 3 or phosphatase 2B. Enzymatic activity of CaN is regulated by the binding of calmodulin (CaM) and calcineurin B subunit that acts as a Ca2+ sensor (Bassel-Duby and Olson, 2006). Once activated, CaN dephosphorylates nuclear factor activater of activated T cells (NFAT), allowing nuclear translocation and subsequent gene expression. Calcineurin signaling can then lead directly to expression of the PPAR and PPARγ coactivator 1α, myogenin, and MyoD (Friday et al., 2003; Long et al., 2007). Therefore, CaN signaling pathway is an important regulator of muscle fiber phenotype. However, CaN expression at the mRNA and protein levels in relation to skeletal muscle type is still not known. Muscle fiber specific expression of CaN may explain the importance of CaN signaling pathway in muscle fiber phenotype control. Therefore, the objective of this study was to characterize porcine CaN mRNA and protein expression in fast and slow skeletal muscles.

MATERIALS AND METHODS

The pig used in this study was raised under commercial conditions and killed at the Purdue University Meat Lab under USDA supervision.
Cloning and Sequencing

Porcine calcineurin gene was cloned by reverse transcription-PCR using RNA extracted from semitendinosus muscle (STM) and brain. Reverse transcription reaction was performed for 50 min at 42°C using 50 ng of random hexamer primer, 200 U of Superscript II RT (Invitrogen, Carlsbad, CA), 50 mM Tris- HCl (pH 8.3), 75 mM KCl, 5 mM MgCl₂, 10 mM dithiothreitol, 500 μM each deoxynucleotide 5’-triphosphate (dNTP), and 1 μg of total RNA. After RT inactivation at 70°C for 10 min, cDNA were purified using Glassmax Spin Cartridges according to manufacturer recommendations (Invitrogen). Full-length porcine CaN was performed in 2 steps; first the full CaN coding sequence and part of 3’ untranslated region of CaN was cloned into the NheI-XhoI cloning site of pcDNA3.1 plasmid (Invitrogen) using the forward Cal5 primer, GCC CTA GCC GAG ATG TCC GAG CCC AAG, and the reverse Cal3 primer, CGC TCG AGT CAG TTT ATA GCC GAG ATG TCC GAG CCC AAG, and the reverse CaN primer, CGC TCG AGT CAG TTT ATA GCC GAG ATG TCC GAG CCC AAG. The PCR reaction was performed in 60 mM Tris- SO₄ (pH 9.1), 18 mM (NH₄)₂SO₄, 1.5 mM MgSO₄, 25 μM each primers, 200 μM each dNTP and 1 unit of Taq polymerase (Elongase enzyme mix; Invitrogen) and 1 μg of STM cDNA. The PCR amplification was performed in a thermocycler (PTC-100, MJ Research Inc., Watertown, MA) using master mix (Promega, Madison, WI); PCR products were electrophoretically separated on 2% agarose gels master mix (Promega, Carlsbad, CA), after PCR amplification using PTC-100, MJ Research Inc., Watertown, MA) using the following conditions: step 1 (2 cycles), denaturation 1 min at 94°C, annealing 1 min at 62°C, extension 2.5 min at 72°C; step 2 (30 cycles), denaturation 1 min at 94°C, annealing 1 min at 62°C, and extension 1.5 min at 72°C. Porcine CaN 5’-end sequences were cloned into the SacI-dIII restriction sites of Bluescript SK+ (Stratagene, La Jolla, CA) after PCR amplification using the primer GCA AGC TTC CCC CCC ACT TCA AAG AGC TCG CGC CGG TGC GGT C and P3 reverse reverse Cal3 primer, CGC TCG AGT CAG TTT ATA GCC GAG ATG TCC GAG CCC AAG, and the reverse CaN primer, CGC TCG AGT CAG TTT ATA GCC GAG ATG TCC GAG CCC AAG. The PCR reaction and amplification conditions were identical to the first step of CaN cloning and were performed on STM and brain cDNA. Two independent PCR products for each construct were cloned and sequenced by the Purdue Animal Sciences Core Sequencing Facility. The BLASTN 2.0.9 and BLASTP 2.0.6 programs (Altschul et al., 1997) were used to perform sequence searches on GenBank (National Health Institute). Porcine DNA and protein sequences were analyzed using DNAsis 2.1 (Hitachi Software Engineering Co. Ltd., Tokyo, Japan).

Analysis of 30-bp Deletion

Primers were designed to flank the deletion site between nt 1412 and 1413 of the determined sequence (forward-AGAGGTGTGCTGTACGGTAAAA, reverse-TGCTGCTGGTCGCGTATGTC). The targeted sequence was amplified by PCR using GoTaq Green master mix (Promega, Madison, WI); PCR products were electrophoretically separated on 2% agarose gels stained with ethidium bromide to determine size. Fragments were excised, purified with the Wizard SV Gel Clean-Up System (Promega), inserted into pGEM-T Easy vectors (Promega), transformed into DH5α Escherichia coli, and cloned. Plasmid DNA was isolated from the bacteria with Pure Yield plasmid miniprep system (Promega) and sequenced at the Virginia Bioinformatics Institute (Blacksburg).

Real-Time PCR

Total RNA was isolated from selected tissues with Trizol (Sigma-Aldrich, St. Louis, MO) according to the manufacturer’s protocol. Isolated RNA was treated with DNase (Ambion, Austin, TX) to eliminate genomic DNA contamination, and RNA concentration was determined with Ribogreen (Invitrogen). Complementary DNA was synthesized using random hexamer primers. Primers designed for real-time quantification are as follows: CaN, forward-AGAGGTGTGCTGTACGGTAAAA, and reverse-TGCTGCTGGTCGCGTATGTC; and 18S, forward-GCTTAATTTGACCCAACAG, and reverse-TGCTCCTCCACAATCAAGAC. Real-time PCR was done using a 7500 Fast Real Time PCR system (Applied Biosystems, Foster City, CA) and Fast Sybr Green master mix (Applied Biosystems). The PCR product size was verified on a 2% agarose gel stained with ethidium bromide.

Immunohistochemistry

Serial muscle cross-sections (10 μm) of the red (RST) and white (WST) portions of the STM and LM of pig muscles were generated at −20°C. To detect CaN-A, sections were fixed 10 min in 4% (wt/vol) paraformaldehyde-PBS solution, then washed thoroughly with PBS buffer. Nonspecific binding was blocked 30 min using normal goat serum diluted (1:32) in PBS buffer. After PBS washing, a CaN-A specific monoclonal antibody (Clone CN-A1, Sigma-Aldrich) was diluted (1:50) in PBS buffer, applied to sections, and incubated overnight at 4°C. After washing in HEPES (0.05 M HEPES, 0.15 M NaCl, pH 7.4) buffer, a Cy2-conjugated AffiniPure F(ab’)2 fragment goat anti-mouse IgG (Jackson Immunoresearch Laboratories, West Grove, PA) was applied to slides and incubated for 1 h at 4°C at a concentration of 4.5 μg/mL in HEPES buffer. Characterization of muscle fiber type was done on nonfixed muscle cross-sections. Primary antibodies used were A4.840 (slow MHC; Hughes and Blau, 1992), 6B8 (2A MHC; Depreux et al., 2000), SC-71 (detects 2A and 2B MHC; Schiaffino et al., 1989). Secondary antibodies were Rhodamine Red-X-conjugated AffiniPure F(ab’)2 fragment goat anti-mouse IgG, and Cy2-conjugated AffiniPure F(ab’)2 fragment goat anti-mouse IgG, Fcγ fragment specific (Jackson Immunoresearch Laboratories).
RESULTS

Porcine Calcineurin-α Gene

Using mouse and human CaN sequences (European Molecular Biology Laboratory accession numbers J05479 and J05480; Kincaid et al., 1990), Cal5 and Cal3 PCR primers were designed to amplify nucleotide sequences from the start codon to nucleotide at position 2181 at the end of the 3' untranslated region of mouse CaN sequence. The expected 2.1-kb amplicon was cloned (Figure 1a). Because the first 5 nucleotides of Cal5 forward primer was mouse sequence, a second PCR step was performed using the forward PCR primer from the 5' untranslated region of the mouse CaN and a specific porcine reverse primer located within porcine CaN coding region to obtain by sequence overlapping the full porcine coding sequence. A potential PCR product of 399 bp was expected. Using STM cDNA, a PCR amplicon of about 400 bp was amplified and cloned (Figure 1b). Brain and STM showed a similar PCR product length (Figure 1b; lanes 1 and 2 vs. lane 3). After sequencing, the 5' end of the CaN clones from brain and STM tissues contained identical sequences (data not shown). Overlapping of cloned sequences from steps 1 and 2 yielded a sequence of 2,179 bp (Figure 2; GenBank accession number AF193515). This sequence was identified homologous to known CaN catalytic subunit. A deletion of 30 bp (Figure 2) was detected in comparison with mouse, human, and bovine CaN sequences (Table 1).

Using standard genetic code for codon translation, a 511 AA putative porcine CaN protein was obtained (Figure 2). Using BLASTP 2.0.6 and DNAsis 2.1, the 511 AA porcine sequence was identified as CaN-A isoform having 99.4% AA identity with rat CaN (Table 1; Perrino et al., 1995). The CaM binding domain of porcine CaN (Figure 2) was located between AA residues 391 and 414 (Kincaid et al., 1988). The calcineurin B subunit binding α helix domain of porcine CaN (Figure 2) was located between AA residues 348 and 368 (Griffith et al., 1995). The autoinhibitory domain of porcine CaN-A resided from AA residues 457 to 481 (Figure 2; Perrino et al., 1995). A putative signal of mRNA polyadenylation was described at nucleotide region 2149 to 2154 (Figure 2; Kincaid et al., 1990).

Calcineurin Expression

Both rat and human are known to have alternative splicing variants of CaN-A (Kincaid et al., 1990).
Therefore, to determine if pigs only express 1 isoform, primers were designed flanking the predicted deletion site at nt 1409 (Figure 2, shaded) and span from nt 1323 to 1577 (Figure 2, arrows). A 255-bp fragment indicates a 30-bp deletion (CaN-Aδ), whereas a 285-bp fragment is the full sequence (CaN-Aα). Surprisingly, 3 bands were observed (Figure 3). However, after extensive effort to isolate and sequence the third band, it was revealed to be nearly a doublet of the 285-bp fragment. The sequence of the CaN-Aα is identical to CaN-Aδ, but with the inclusion of the sequence CTGTTGAG-GCTATTGAGGCTGATGAAGCTA in the deletion site. Liver, spleen, and kidney express only CaN-Aδ. By contrast, brain, heart, and skeletal muscles express CaN-Aδ and CaN-Aα. Although not quantitative, the ratio of 285- to 255-bp fragments appears to be associated with muscle fiber type. An interesting observation was the presence of a point mutation at bp 1393 from G to A. This mutation was found in approximately one-half of the sequences, indicating this pig is homozygous for this allele. However, this mutation does not result in a difference in the AA sequence and is likely of no consequence.

A second set of primers were designed to measure total CaN expression. These primers produced a single PCR product in all tissues (data not shown). Real-time PCR analysis showed that CaN is expressed in all tissues assayed (Figure 4).
**Immunohistochemistry**

Fluorescence intensity from CaN staining was brightest in fibers around the periphery of the fasciculi of RST. Using monoclonal antibodies against each myosin heavy chain (MyHC) isoforms, the reactivity of CN-A1 clone was least in slow fibers for all skeletal muscles (Figures 5b, 6b, and 7b). However, CN-A1 clone reac-

![Image of nucleotide sequence and deduced AA sequence of partial porcine calcineurin (CaN)-A cDNA.](image-url)

*Figure 2 (Continued).* Nucleotide sequence and deduced AA sequence of partial porcine calcineurin (CaN)-A cDNA. The nucleotide residue numeration follows a 5’ to 3’ direction, beginning with the first residue. The AA-deduced sequence and numeration are shown below the nucleotide sequence and start by initial methionine AA. The asterisk indicates a stop codon. Bold AA are located on the putative CaN-B binding domain. Underlined AA are located on the putative calmodulin binding domain. Bold and italic AA residues are localized in the putative autoinhibitory domain. Bold nucleotides correspond to CaN sequence cloned into Bluescript SK+ (Stratagene, La Jolla, CA) as CaN transcription plasmid. Shaded nucleotides delimit the 30-bp deletion region. Arrows indicate the location of primers flanking the 30-bp deletion region. The underlined nucleotides correspond to the putative signal of mRNA polyadenylation.
Table 1. Comparison of porcine calcineurin-α sequence with that of other species

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Figure 3. The PCR products generated using primers designed to flank the 30-bp deletion observed during sequencing. Fragments of 285 and 255 bp were produced with size confirmed by sequencing. Although a third band is visible in brain and muscle tissues under longer running times in greater percentage agarose gels, sequence analysis showed only the presence of a 285- and a 255-bp fragment. WST = white portion of semitendinosus muscle; RST = red portion of semitendinosus muscle.

Figure 4. Real-time PCR analysis of calcineurin presence in different porcine tissues. All values are normalized to 18s expression and reported relative to calcineurin expression in brain. WST = white portion of semitendinosus muscle; RST = red portion of semitendinosus muscle.
tivity was associated with fibers expressing MyHC 2A, 2X, and 2B (Figures 5, 6, and 7).

**DISCUSSION**

Using CaN-α isoform-specific PCR primers to amplify porcine CaN-A from STM cDNA, we cloned a partial cDNA that contains the entire predicted open reading frame of CaN-A. From the nucleotide sequence, a 30-bp deletion resulting in loss of 10 AA (T V E A I E A D E A) located between the CaM binding domain and C terminal region where autoinhibitory domain is localized, was observed. There are 2 major classes of catalytic subunit isoforms: CaN-Aα or type 1 and β or type 2 (Kincaid et al., 1991). The CaN-Aβ isoform differs from the α isoform by the presence of a proline-rich region of the amino-terminal end of the molecule (Kincaid et al., 1991). Originally identified in mouse and human brain tissues (Kincaid et al., 1990), CaN-Aα is known to generate a slice variant by deletion of a 30-bp sequence designated CaN-Aδ (Perrino et al., 1992). This deletion has been described in humans and mice and is likely due to alternative splicing (Kincaid et al., 1990). The full-length CaN-A is denoted CaN-1α (Kincaid et al., 1990, 1991; Perrino et al., 1992). The CaN-Aδ is present in smaller amounts in brain than the full length CaN-1α (Kincaid et al., 1991). By targeting the deletion region, we do show the presence of CaN-Aα and CaN-Aδ in brain, heart, and skeletal muscles. The function of this deletion has not been described, although there may be regulatory implications. The close vicinity of the deletion to autoinhibitory domain might affect regulatory properties; however, CaN-Aδ enzymatic activity has been shown to be under autoinhibitory domain regulation (Kincaid et al., 1990, 1991; Perrino et al., 1995). In humans, it has been shown that cleavage of calcineurin by calpain I C-terminal to the autoinhibitory domain significantly increases phosphatase activity (Liu et al., 2005). This deletion may allow for tissue-specific regulation of calcineurin activity by enhancing/preventing cleavage by a protease. Regardless, the biological significance of AA deletion on CaN-Aδ enzymatic activity is still unknown. Also interesting, the apparent ratio of CaN-Aα to CaN-Aδ appeared to be associated with muscle fiber type. Although these data are not quantitative, this does lead us to speculate a potential relationship between specific splice variants and muscle fiber type-specific gene expression.

The immunological localization of CaN-A mainly in cytoplasm of fast skeletal muscle fiber is interesting in relation to the hypothesis that CaN-A controls the slow muscle fiber phenotype (Chin et al., 1998; Hughes, 1998). In agreement with these data, Parsons et al. (2003) showed increased calcineurin expression...
Figure 6. Immunolocalization of calcineurin-A (a), slow myosin heavy chain (MyHC; b), 2A MyHC (c), 2A and 2X (d), and 2B MyHC (e) on muscle sections (white semitendinosus). Phase field (f). Bar = 50 μm. Color version available in the online PDF.

Figure 7. Immunolocalization of calcineurin-A (a), slow myosin heavy chain (MyHC; b), 2A MyHC (c), 2A and 2X (d), and 2B MyHC (e) on muscle sections (LM). Phase field (f). Bar = 50 μm. Color version available in the online PDF.
and activity in mice. By contrast, Oh et al. (2005) demonstrated CaN-A protein content is greater in mouse soleus than extensor digitorum longus muscle. These differences may be due to the specificity of the antibody used. In addition to CaNα, there is also a β isoform. Indeed, Parsons et al. (2003) identified 3 isoforms in fast muscle, whereas slow fibers only expressed 1. The antibody used in this study is specific for the α isoform in human, rat, and bovine. However, it is possible that it may cross-react with other isoforms in the pig. Calcineurin is known to dephosphorylate nuclear factor of activated T-cells (NFAT) in the cytoplasm and co-translocate to nucleus, presumably to sustain NFAT biological activity in nucleus. Therefore, it is possible that in slow muscle fibers, CaN is mainly localized in the nucleus. However, increased quantity of autofluorescence in nuclei of our negative control slides did not allow us to confirm this first hypothesis.

There is emerging evidence demonstrating CaN is actively involved in regulating muscle fiber phenotype. Cyclosporin inhibition of CaN activity induces a slow to fast transition fiber phenotype in red soleus (Chin et al., 1998) but has no effect on fiber type in the fast plantaris (Dunn et al., 1999). However, induction of CaN activity in the overloaded plantaris induced muscle fiber hypertrophy, and after 4 wk, the transcription of slow MyHC mRNA. Targeted suppression of muscle CaN in MyoD-cre and MCIP-Flox mice resulted in elimination of slow fibers without affecting oxidative capacity, myoglobin content, or abundance of mitochondria (Oh et al., 2005). These data indicate that CaN is involved in regulation of slow MyHC expression and may affect slow fiber phenotype. Fiber type-specific regulation of CaN signaling may also be mediated by the CaN binding protein cascarin. Genetic ablation of the fast-fiber specific calscarin-2 resulted in a distinct shift from fast fibers to slow, oxidative fibers (Frey et al., 2008).

Porcine CaN-A gene cloning allowed us to show that CaN-A expression in skeletal muscle is potentially regulated at the posttranscriptional level. Therefore, an increased quantity of CaN-A expression in fast fibers may underscore the importance of this Ca2+/CaM-dependent Ser/Thr phosphatase in all muscle fiber types. Subsequent communication at the cellular level between P3K and Ca2+ immervation-dependent signaling pathways is unclear. In this context, the significance of a different CaN-A mRNA length is not known. Therefore, the role of CaN in controlling muscle fiber phenotype as recently suggested is not entirely clear and needs further investigation.

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