CaMKII Protein Expression and Phosphorylation in

Mouse Skeletal Muscle Following Atrophy and Hypertrophy

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May, 2012

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The maintenance of skeletal muscle mass is vital for life, and elucidation of the molecular mechanisms that control this process is a critical first step towards the development of pharmaceutical treatments for muscle wasting disorders. Intracellular Ca^{2+} is a regulator of muscle growth, yet surprisingly the signaling proteins by which Ca^{2+} regulates this function remain largely unknown. The serine/threonine kinase, Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), is one of the Ca^{2+}-regulated proteins found in muscle. The goal of this study was to determine whether the phosphorylation or protein expression of any of the CaMKII isoforms present in skeletal muscle (i.e. CaMKIIβ_M, γ or δ) is altered in response to muscle atrophy or hypertrophy. Male, CD-1 mice (~6-8 weeks old) underwent unilateral denervation of the hindlimb to induce atrophy, and the plantaris muscles removed 1, 3, 7, 10 and 14 days later. In addition, mice underwent unilateral ablation of the gastrocnemius and soleus muscles to induce hypertrophy and the plantaris muscles removed 1, 3, 7 and 10 days later. Consistent with previous studies, denervation induced a time-dependent decrease in muscle weight after 3 days, and ablation induced a time-dependent increase in muscle weight after 3 days. To assess alterations in CaMKII (Thr286/287) phosphorylation and expression western blot analyses were performed. Denervation elicited a significant decrease in CaMKIIβ_M expression after 3 days, but

an increase in CaMKIIγ and CaMKIIδ after 10 days. Phosphorylation of CaMKIIγ was decreased after 7 days, while there were no alterations in CaMKIIβ_M or CaMKIIδ. Ablation induced a significant decrease in CaMKIIβ_M expression after 7 days, an increase in CaMKIIδ after 7 days, but no change in CaMKIIγ. Phosphorylation of CaMKIIγ was significantly decreased after 1 day, while there were no changes in CaMKIIβ_M or CaMKIIδ. Collectively, these results suggest a differential and complex regulation of CaMKII isoforms during alterations in muscle mass. In summary, this study demonstrated that in mouse skeletal muscle CaMKII isoforms are differentially phosphorylated and expressed during denervation-induced atrophy and synergist ablation-induced hypertrophy. These findings underscore the necessity of examining each CaMKII isoform separately in order to determine its possible role in regulating muscle mass.

CaMKII Protein Expression and Phosphorylation in Mouse Skeletal Muscle Following Atrophy and Hypertrophy

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Brett Williams

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in Mouse Skeletal Muscle Following Atrophy and Hypertrophy

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CHAPTER 1: BACKGROUND AND SIGNIFICANCE

Skeletal muscle plays an important role in a number of critical functions in the human body including locomotion, respiration, and metabolism; and a decrease in muscle mass can impair all of these functions leading to decreased quality of life and increased mortality. Loss of muscle mass can occur for many reasons and from various circumstances, including the following conditions: 1) living a sedentary lifestyle or muscle disuse (Yan et al., 2010; Evans, 2010; Lecker et al., 2004); 2) cancer cachexia (Muscaritoli et al., 2006; Lecker et al., 2003; Barber et al., 1999; Evans, 2010); 3) aging-induced sarcopenia (Evans, 2010; Thompson and Gordon, 2006); and 4) loss/deficits in nerve function (Castro et al., 1999; Giangregorio and McCartney, 2006). Due to the large number of individuals affected by decreases in muscle mass and the severe consequences incurred when muscle wasting is not reversed, determination of the cellular and molecular factors underlying the regulation of skeletal muscle mass is a critical undertaking that could lead to the development of pharmaceutical treatments for muscle wasting disorders.

Hypertrophy and Skeletal Muscle

The size of skeletal muscle increases via hypertrophy, a process characterized by an increase in the cross-sectional area of muscle fibers. This should not be confused with hyperplasia which is an increase in the number of muscle fibers. Conditions or events that may lead to muscle hypertrophy include resistance exercise, anabolic steroids, hormones (e.g. growth hormone, testosterone, epinephrine), growth factors (human growth factor, insulin-like growth factor (IGF-1)), functional overload induced by surgical ablation of synergist muscles, etc. (Baechle and Earle, 2008). In this study, we will induce hypertrophy through synergist ablation.

Synergist muscles are defined as a group of muscles working together to perform a common movement about a joint. We will stimulate functional overload in the plantaris muscle of mice through ablation of the soleus and gastrocnemius. By surgically removing these two muscles, the functional load on the plantaris is increased, thus inducing hypertrophy (Roy and Edgerton, 1995). This procedure will be described in detail in the "*Materials and Methods*" section.

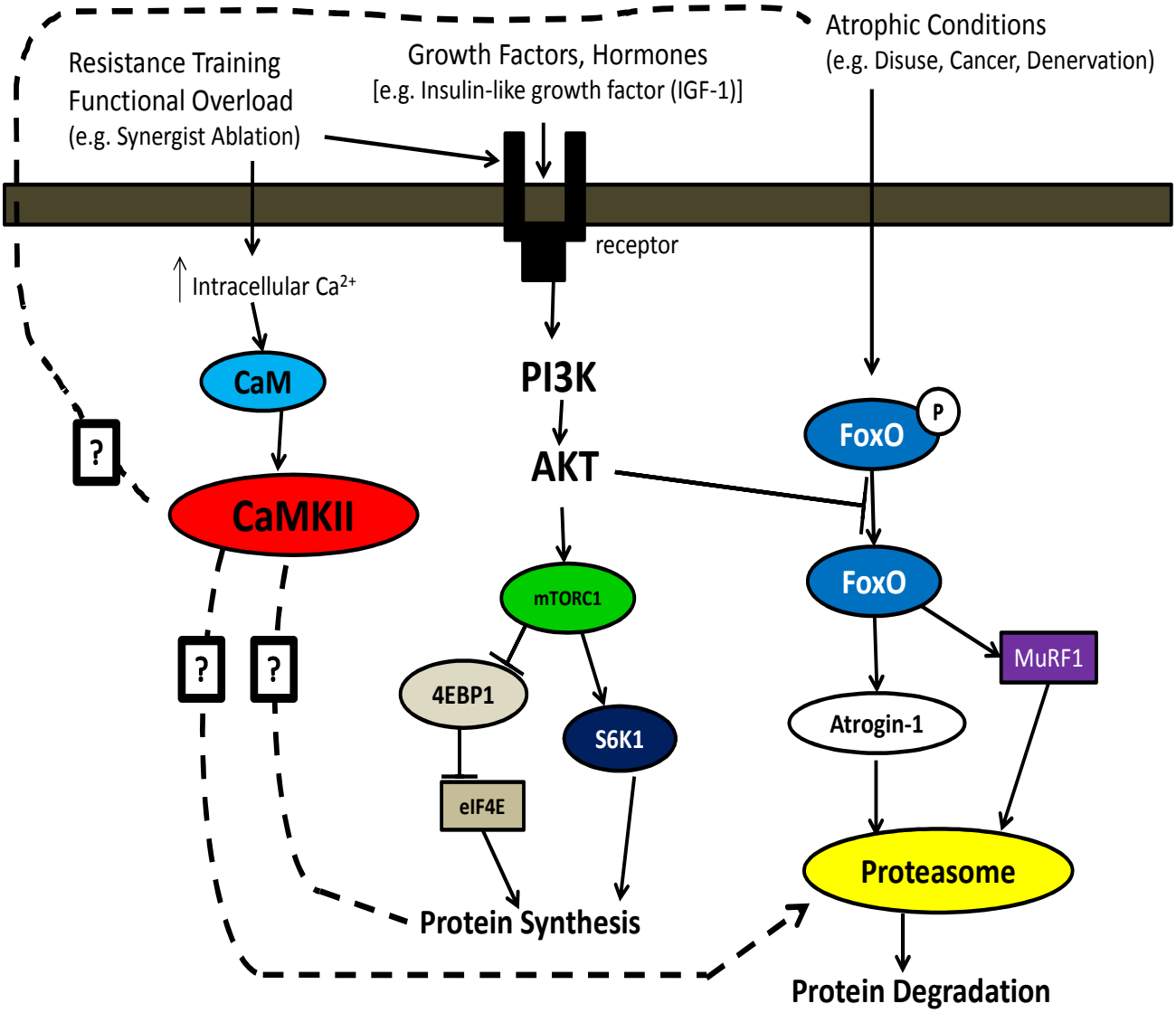
Atrophy and Skeletal Muscle

In a general sense, muscle atrophy could be considered the opposite of muscle hypertrophy. Skeletal muscle atrophy is defined as a decrease in muscle mass. It is caused by an increase in protein degradation and a decline in protein synthesis (Jones et al., 2004). Atrophy can have detrimental effects on the human body due to the involvement of skeletal muscle with movement (Thomson and Gordon, 2006), glucose and protein metabolism (Lightfoot, 2009) respiration (Muscaritoli, 2006) and immune response (McArdle et al., 2004; Hartl, 1996; Juretic et al., 1994; Spittler et al., 1995). In this study, muscle atrophy will be induced through resection of the sciatic nerve. The sciatic nerve is a very large spinal nerve that innervates a large number of muscles in the posterior leg, including the gastrocnemius, soleus, and plantaris muscles. This procedure will be described in detail in the "*Materials and Methods*" section.

Intracellular Signaling Proteins Regulating Skeletal Muscle Protein Synthesis

Skeletal muscle mass is regulated by the balance between protein synthesis and protein degradation. Under steady-state conditions, the rate of protein synthesis equals the rate of protein breakdown. One cellular pathway that is a major contributor for the maintenance of protein synthesis is the phosphoinositide 3-kinase (PI3K)/Akt signaling pathway (Sandri, 2008)

(Figure 1). It begins with the growth promoting factor insulin-like growth factor 1 (IGF-1), which has been linked to muscle hypertrophy when overexpressed (Musaro et al., 2001) and following functional overload in mice (McCall et al., 2003). IGF-1 stimulates PI3K which then phosphorylates Akt, a serine/threonine protein kinase that plays a role in many cellular processes. Once activated, Akt phosphorylates and activates the mammalian target of rapamycin (mTOR). Studies have shown that when rapamycin (an inhibitor of mTOR) is introduced to the muscle, hypertrophy is downregulated (Bodine et al., 2001b; Pallafacchina et al., 2002). Studies have also shown that mTOR induces the phosphorylation of eIF4E binding protein (4E-BP1), which promotes translation initiation of 5'-cap mRNAs (Shah et al., 2000; Takata et al., 1999). mTOR also phosphorylates the 70 kDa ribosomal protein S6 kinase (p70S6K1) which promotes the translation of various ribosomal proteins and elongation factors (Terada et al., 1994) and, when p70S6K1 is knocked out in mice, the response to Akt and IGF1 is downregulated (Ohanna et al., 2005).



<u>Figure 1</u>: Cell Signaling Model Representing Intracellular Proteins and Their Roles in Protein Degradation and/or Protein Synthesis. The dashed lines going to and coming from CaMKII show that the role of CaMKII in protein synthesis and protein degradation is unclear. [Legend: CaM=calmodulin; CaMKII=Ca^{2+}/calmodulin-dependent protein kinase; PI3K=phosphatidylinositol 3 kinase; Akt=protein kinase B; mTORC1=mammalian target of rapamycin; 4EBP1=4E-binding protein-1; S6K1=S6 kinase 1; eIF4E=eukaryotic translation initiation factor 4E; FoxO=forkhead box type O; MuRF 1=muscle RING-finger protein-1; IGF-1=insulin-like growth factor-1]

Intracellular Signaling Proteins Regulating Skeletal Muscle Protein Degradation

Similar to hypertrophy, muscle atrophy is also regulated by many molecular and cellular mechanisms. One of these is through the ubiquitin-ligases atrogin-1 and muscle RING-finger protein-1 (MuRF1) and activity of the proteasome (Figure 1). When atrogin-1 and MuRF1 are upregulated, they are both associated with an increase in protein degradation through the ubiquitin-proteasome system (Bodine et al., 2001a; Gomes et al., 2001). When these genes are knocked out in mice, denervation-induced muscle atrophy is limited (Bodine at al., 2001a).

The proteasome is the primary site of intracellular protein degradation and regulates the levels of proteins in our bodies by breaking them down (Ciechanover, 1994). Molecules called ubiquitin are used to tag the proteins that are to be degraded (Bodine et al., 2001a). Under normal circumstances, this chain of events is a necessary component for the degradation and recycling of old or misfolded proteins (Ciechanover, 1994). When these proteolytic pathways become hyperactivated due to disease or other abnormal conditions, excessive muscle degradation may occur (Muscaritoli et al., 2006).

Another important molecule associated with atrophy is FoxO, a member of the forkhead family of transcription factors. One study has shown that mice with upregulated FoxO1 had decreased muscle mass (Kamei et al., 2004; Southgate et al., 2007) and an upregulation of FoxO3 has been shown to promote atrogin-1 and MuRF1 activity and protein degradation (Sandri et al., 2004). Whereas FoxO has been shown to upregulate atrogin-1 and MuRF1, Akt negatively regulates the function of FoxO (Lee et al., 2004; Sandri et al., 2004). Therefore, Akt is not only associated with hypertrophy but is also believed to help downregulate atrophy.

Ca^{2+}/Calmodulin-Dependent Protein Kinase II (CaMKII)

Intracellular Ca^{2+} is a key regulator of numerous processes in skeletal muscle, including metabolism and growth (Djakovic et al., 2009; Al-Shanti and Stewart, 2009; Chin, 2004), yet surprisingly, the intracellular signaling mechanisms by which Ca^{2+} regulates these critical functions still remain largely unknown. The multi-functional Ca^{2+}/calmodulin-dependent protein kinases (CaMKs) are a group of serine/threonine protein kinases that are dependent on Ca^{2+}/calmodulin for activation (DeKoninck and Schulman, 1998). Numerous isoforms of CaMK have been identified in mammalian cells including Ca^{2+}/calmodulin-dependent protein kinase kinase α and β (CaMKKα and CaMKKβ), Ca^{2+}/calmodulin-dependent protein kinase I α, β, γ, and δ (CaMKIα, CaMKIβ, CaMKIγ, and CaMKIδ), Ca^{2+}/calmodulin-dependent protein kinase II α, β, γ, and δ (CaMKIIα, CaMKIIβ, CaMKIIγ, and CaMKIIδ) and Ca^{2+}/calmodulin-dependent protein kinase IV (CaMKIV) (Braun and Schulman, 1995; Fluck et al., 2000; Hudman and Schulman, 2002; Kamata et al., 2007; Soderling et al., 2001). All of these isoforms are categorized as multi-functional CaMKs due to their ability to phosphorylate multiple substrates.

Not all of the CaMK isoforms are found in skeletal muscle. For example, CaMKIV is not present in skeletal muscle (Akimoto et al., 2004). However, the isoforms CaMKKα, CaMKIα, β, δ and CaMKIIβ_M, γ, and δ have been detected in skeletal muscle (Chin, 2004; Fluck et al., 2000; Hudman and Schulman, 2002; Stephens et al., 2010; Witczak et al., 2007).

Each isoform of CaMK is differentially regulated by increases in intracellular Ca^{2+} and the binding of the Ca^{2+}/calmodulin complex. Figure 2 depicts how the Ca^{2+}/calmodulin complex directly activates CaMKK which in turn phosphorylates CaMKI and CaMKIV (Ishikawa, 2003; Tokumitsu, 2004). For complete activation of CaMKI and CaMKIV, Ca^{2+}/calmodulin must also bind to these two isoforms. In contrast, CaMKII relies solely on Ca^{2+}/calmodulin for activation

(Saucerman and Bers, 2008; Stefan, 2008). Figure 3 illustrates the autophosphorylation cycle of CaMKII. Once Ca^{2+}/calmodulin binds to CaMKII, the kinase can activate downstream effectors through phosphorylation. This process is known as Ca^{2+}/calmodulin-dependent activity (Hudmon and Schulman, 2002). From here, CaMKII may then be autophosphorylated at the threonine-286/287 residue. After autophosphorylation occurs, CaMKII can now remain active independent of the Ca^{2+}/calmodulin complex (Anderson et al., 1994; Fluck et al., 2000; Shiaffino and Reggiani, 1996) which also enables it to signal to downstream effectors. This process is known as Ca^{2+}/calmodulin-independent activity. Deactivation of CaMKII is mediated through Ca^{2+}/calmodulin dependent protein kinase phosphatase (CaMKPase) (Ishida et al., 1998).

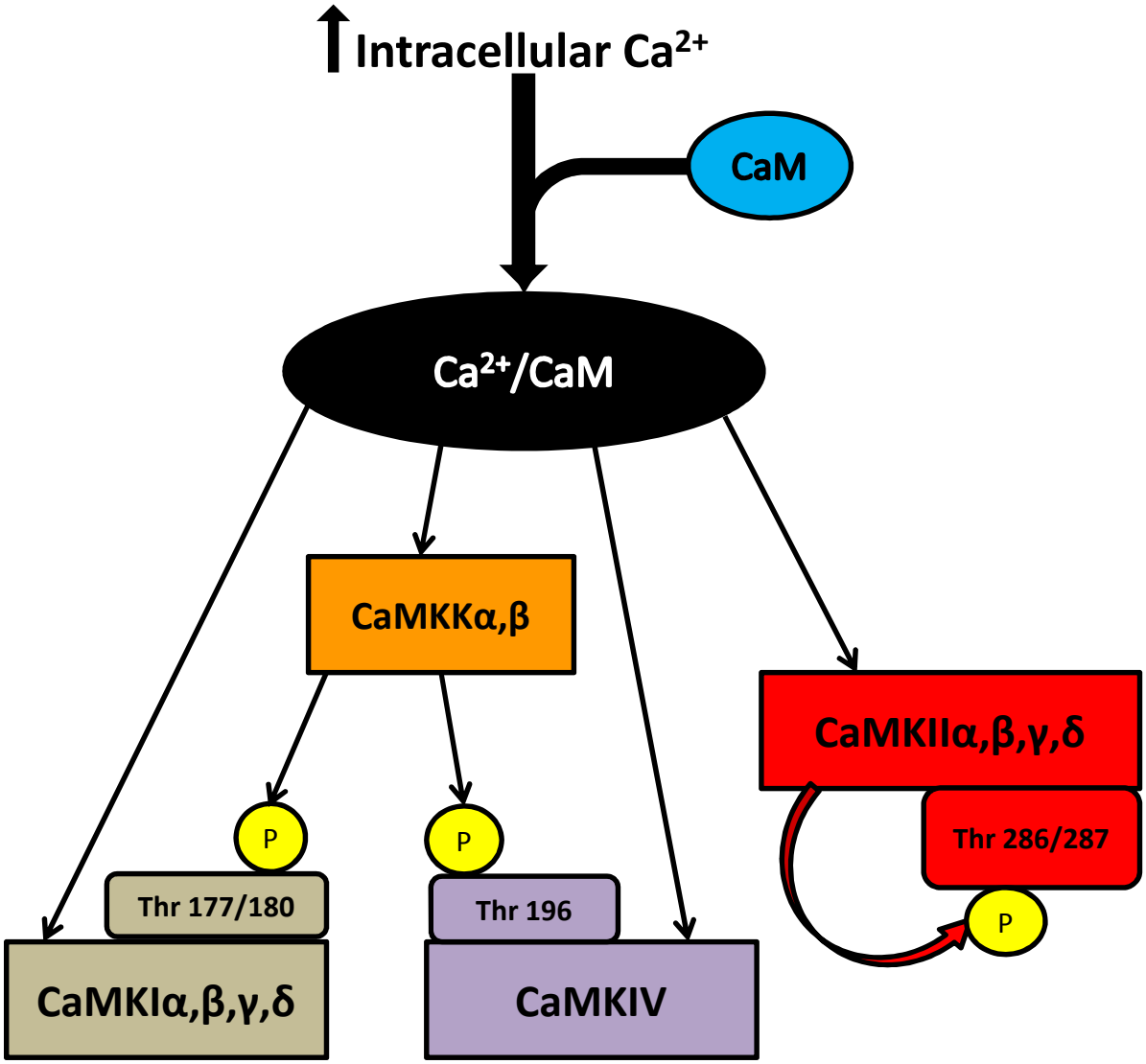


Figure 2: **Differential Regulation of the Ca^{2+}/Calmodulin-Dependent Kinase (CaMK) Isoforms by Ca^{2+}/Calmodulin (Ca^{2+}/CaM).** Ca^{2+}/calmodulin-dependent protein kinase kinase α and β (CaMKK), Ca^{2+}/calmodulin-dependent protein kinase I α, β, γ, and δ (CaMKI), Ca^{2+}/calmodulin-dependent protein kinase II α, β, and γ (CaMKII) and Ca^{2+}/calmodulin-dependent protein kinase IV (CaMKIV). The "P" in the yellow oval illustrates that phosphorylation is taking place.

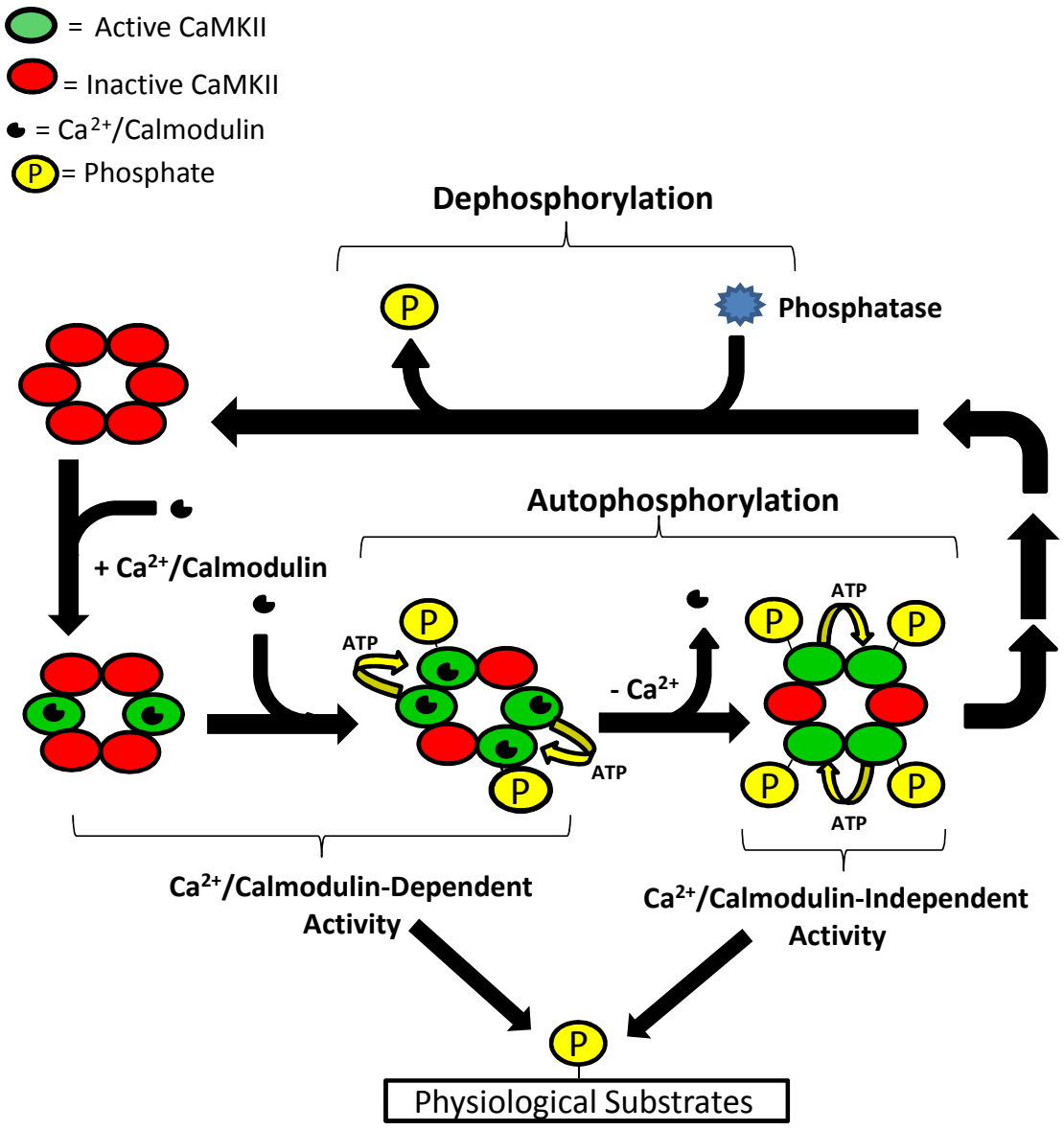


Figure 3: Ca^{2+}/Calmodulin-Dependent Protein Kinase II (CaMKII) Autophosphorylation Cycle. Figure adapted from Hudmon A, Schulman H. Structure-function of the multifunctional Ca2+/calmodulin-dependent protein kinase II. Biochem J. 364(Pt. 3): 593-611, 2002.

Intriguingly, a few studies have now suggested that CaMKII may play a key role in the regulation of skeletal muscle mass and protein degradation (Chin, 2004; Djakovic et al., 2009; Witczak unpublished findings, 2010). As shown in Figure 4, skeletal muscle taken from aged rats (27 months) shows a ~2.5-fold increase in the protein expression of CaMKIIγ when compared to the skeletal muscle of young rats (4 months) (Chin, 2004). Since Thomson and Gordon (2005 and 2006) have previously shown that aging is accompanied with a decrease in muscle mass (sarcopenia), these findings may indicate that CaMKIIγ is involved in aging-induced muscle atrophy. In another study performed by Chin et al. (2004), CaMKIIγ protein expression was measured in the soleus muscle of young rats 20 days after denervation surgery (i.e. unilateral severing of sciatic nerve). Figure 5 shows that CaMKIIγ expression increased nearly 3-fold in the denervated soleus when compared to control. These findings may suggest a role for CaMKIIγ in denervation-induced muscle atrophy. To date, no studies have been performed to suggest a role for CaMKIIβ_M or CaMKIIδ in skeletal muscle atrophy.

CaMKII has also been implicated as an intracellular player in proteasome activity in non-muscle cells (Djakovic et al., 2009). Although the focus of this project is on CaMKII in skeletal muscle, it is worth noting the data linking CaMKII activity and protein degradation in non-muscle cells. In Figure 6, there is a 30% decrease in protein levels in GFP-tagged cells expressing constitutively active CaMKII (i.e. CaMKII T286D) when compared to control protein levels (31% to 61%, respectively). The decreases in protein levels were due to an increase in proteasome activity. In Figure 7, constitutively active CaMKII in rat hippocampus neurons provides similar results to that of Figure 6. The bar graph in Figure 7 shows a 14% increase in proteasome activity in neurons expressing constitutively active CaMKII when compared to control levels. To confirm that CaMKII was solely causing the increase in proteasome activity,

western blots were performed on the proteasomes (Figure 7). The results indicate that total proteasome levels were very similar in both control neurons and neurons with constitutively active CaMKII, demonstrating that CaMKII increases proteasome activity.

In contrast to the growing amount of data suggesting a role for CaMKII in the regulation of skeletal muscle atrophy and proteasome activity, to date there is no published evidence suggesting a role for CaMKII in the regulation of skeletal muscle hypertrophy. However, preliminary results from our lab now show an initial decrease in the levels of CaMKIIβ_M, γ, and δ in the plantaris muscle of male mice after synergist ablation surgery (i.e. unilateral functional overload) (Figure 8). When the overloaded plantaris muscle of mice 1 and 3 days post-surgery were compared to the sham-operated plantaris from the same mice, the levels of CaMKII were 50% and 25% lower in the overloaded plantaris, respectively. These results show that hypertrophic conditions in skeletal muscle cause a decrease in the expression of CaMKII. Collectively, these results show that the intracellular protein kinase CaMKII is affected by muscle atrophy and muscle hypertrophy, suggesting that CaMKII could be a key regulator of protein synthesis, protein degradation and/or muscle mass in skeletal muscle.

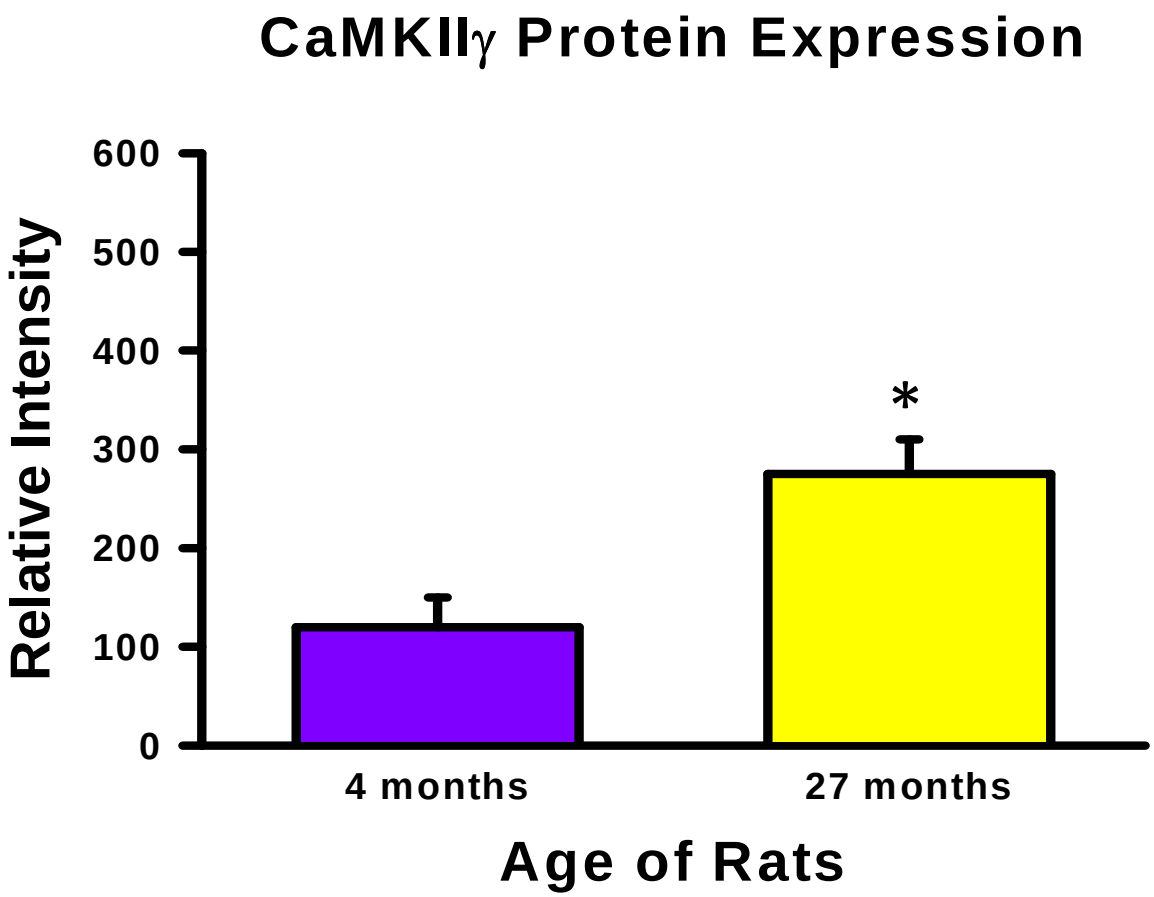


Figure 4: CaMKIIγ Protein Expression Is Increased in Aged Rat Skeletal Muscle. The figure was adapted from Chin ER. The Role of Calcium and Calcium/Calmodulin-Dependent Kinases in Skeletal Muscle Plasticity and Mitochondrial Biogenesis. Proceedings of the Nutrition Society. 63(2): 279-287, 2004. The "*" indicates a statistically significant difference ($P<0.05$) compared to 4 months.

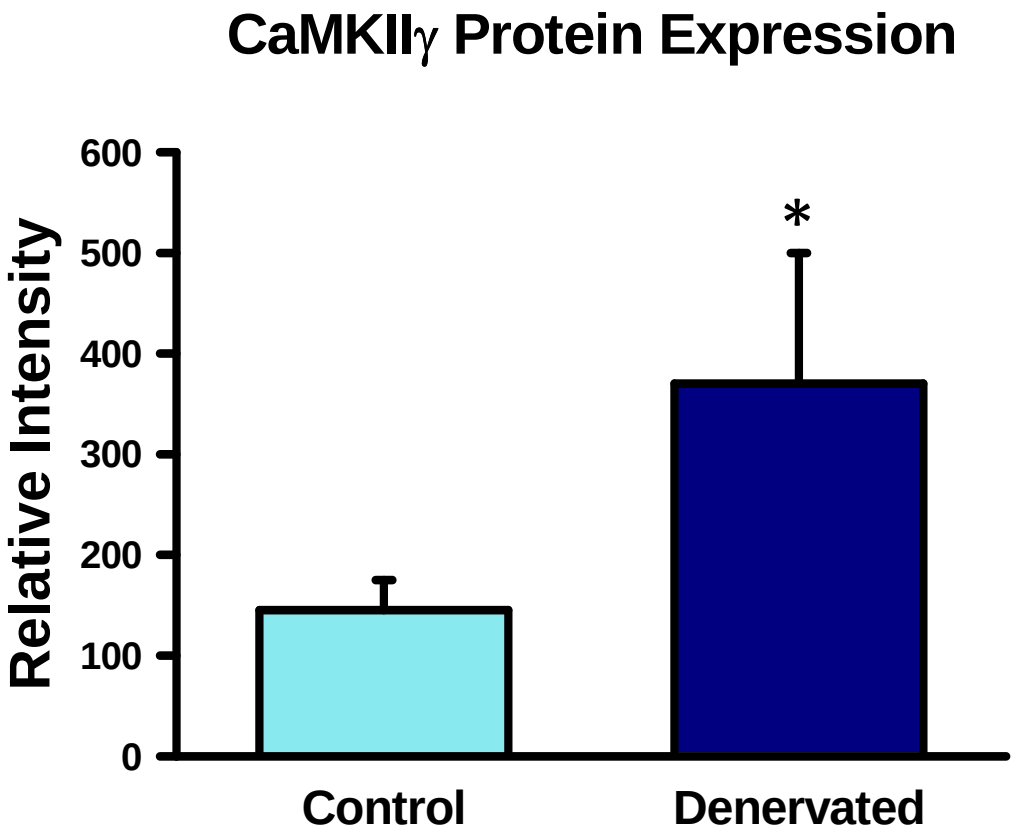


Figure 5: **CaMKIIγ Protein Expression is Increased in Rat Soleus Muscle Following Denervation**. The figure was adapted from Chin ER. The Role of Calcium and Calcium/Calmodulin-Dependent Kinases in Skeletal Muscle Plasticity and Mitochondrial Biogenesis. Proceedings of the Nutrition Society. 63(2): 279-287, 2004. The "*" indicates a statistically significant difference ($P<0.05$) compared to control muscles.

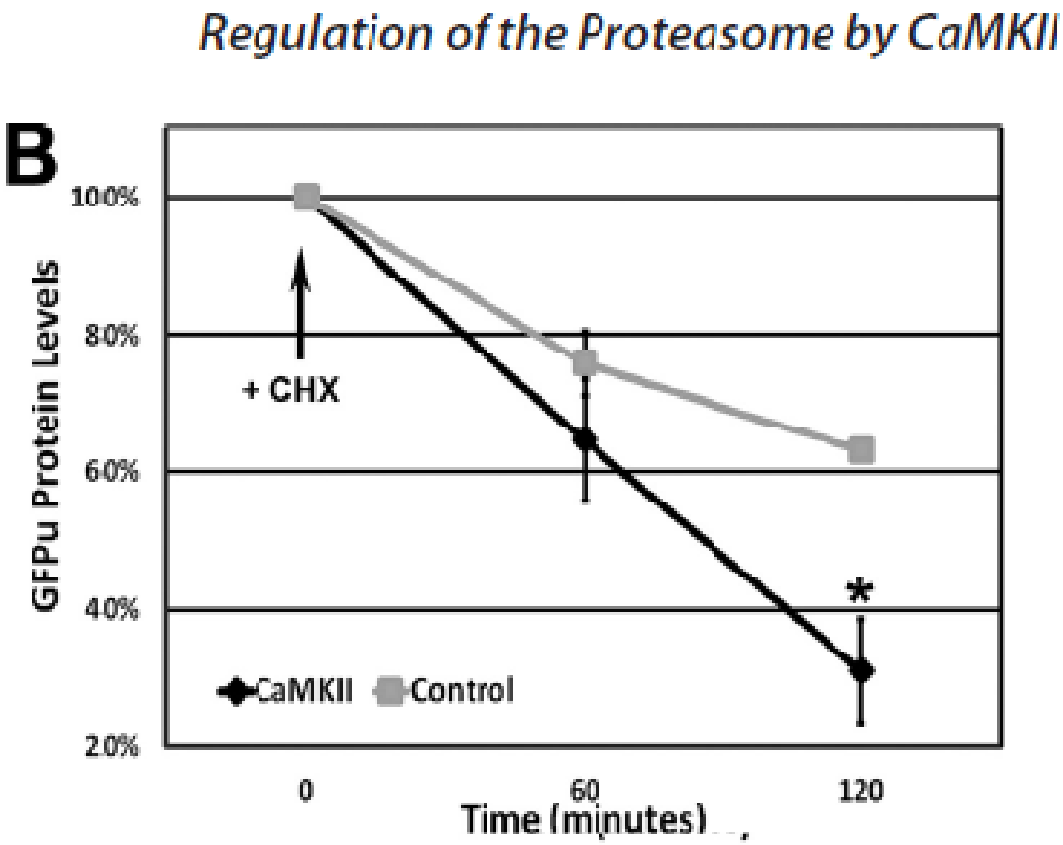


Figure 6: **Overexpression of Constitutively Active CaMKII in HEK293T cells Increases Protein Degradation.** Cells were labeled with GFP and an ubiquitin-proteasome reporter and co-transfected with constitutively active CaMKIIα. Total protein levels were measured and compared to control levels. Cells expressing CaMKII T286D contained about 30% lower protein levels. The protein synthesis inhibitor cycloheximide (CHX) was introduced to the cells to prevent increases in protein synthesis. The figure was taken from Djakovic SN et al. Regulation of the proteasome by neuronal activity and calcium/calmodulin protein kinase II. J Biol. Chem. 284(39): 26655-26665, 2009. The "*" indicates a statistically significant difference ($P<0.05$) compared to control cells.

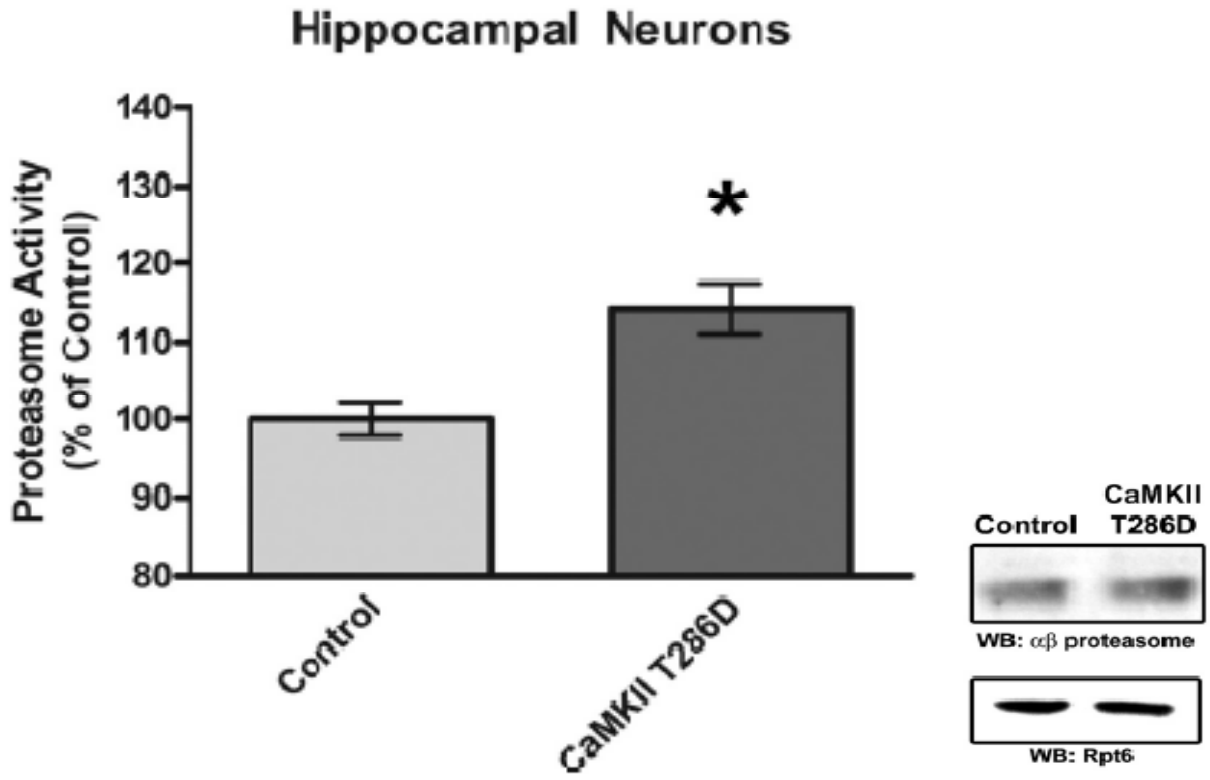
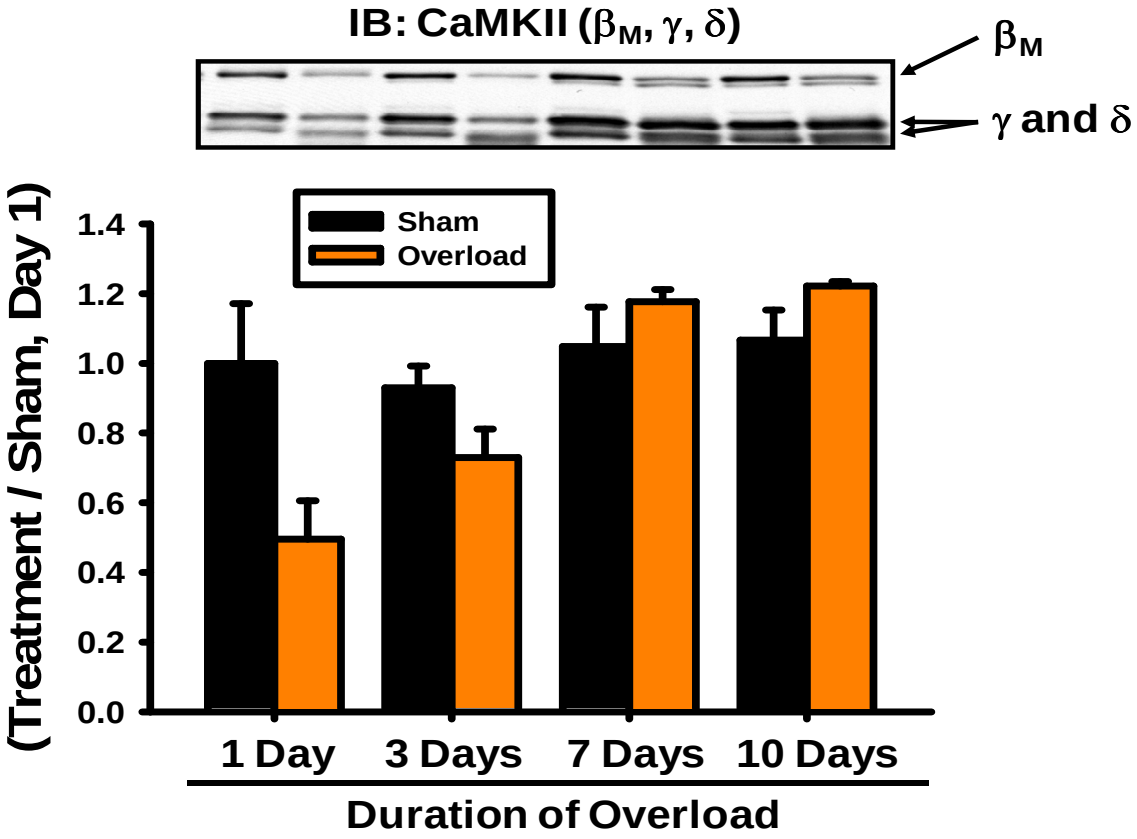


Figure 7: Overexpression of Constitutively Active CaMKII in Rat Hippocampal Neurons Stimulates Proteasome Activity. The figure was taken from Djakovic SN et al. Regulation of the proteasome by neuronal activity and calcium/calmodulin protein kinase II. J Biol. Chem. 284(39): 26655-26665, 2009. The "*" indicates a statistically significant difference ($P<0.05$) compared to control cells.



<u>Figure 8:</u> Overload-Induced Skeletal Muscle Hypertrophy in Mice Induces a Time-Dependent Decrease in CaMKIIβ_M, γ, and δ Protein Expression. Witczak CA. Unpublished observations, 2010. (N = 2-3 muscles/group).

Hypothesis

Previous studies in rats have shown that the protein expression of CaMKIIγ increases and remains elevated following denervation-induced muscle atrophy (Chin, 2004). In addition, preliminary experiments in mice have shown that CaMKII protein expression initially decreases when muscle hypertrophy is induced by ablation surgery (Witczak CA, unpublished observations, 2010). Therefore, we hypothesize that following muscle denervation, as protein degradation increases and protein synthesis decreases, CaMKII protein expression and phosphorylation (activation) will increase and remain elevated. In addition, we hypothesize that following muscle overload, as protein degradation decreases and protein synthesis increases, CaMKII protein expression and phosphorylation (activation) will decrease and then return to baseline levels as protein synthesis slows.

Objectives

The overall goal of this project is to determine whether one or more of the CaMKII isoforms present in skeletal muscle is altered in response to hypertrophic or atrophic stimuli and the possible time dependency of those changes. To address this goal, we propose to stimulate mouse skeletal muscle to hypertrophy via unilateral synergist ablation surgery and examine CaMKII (Thr286/287) phosphorylation and protein expression 1, 3, 7, and 10 days later. In addition, we will also stimulate mouse skeletal muscle to atrophy via unilateral hindlimb denervation and examine CaMKII (Thr286/287) phosphorylation and protein expression 1, 3, 7, 10, and 14 days later.

Expected Results

The results from previous studies performed on CaMKII allow us to make predictions about CaMKII protein expression and phosphorylation in skeletal muscle and how they are affected by conditions that cause muscle atrophy (denervation) and muscle hypertrophy (overload). We expect a decline in plantaris muscle weight 3, 7, 10 and 14 days after denervation surgery. Since we expect CaMKII activity to increase during atrophic conditions, we should see CaMKII (Thr286/287) phosphorylation and expression increase initially 1 day after denervation and then increase to levels much higher than control 3,7,10,and 14 days following denervation surgery.

Following synergist ablation surgery, we expect a significant increase in plantaris muscle weight after 3, 7, and 10 days. During conditions of hypertrophy, we expect CaMKII (Thr286/287) phosphorylation and expression to significantly decrease in overloaded muscle 1 day following surgery. Following day 1, we expect a gradual increase in CaMKII phosphorylation and expression in 3, 7 and 10 day overloaded muscle following synergist ablation surgery. By days 7 and 10, we do not expect CaMKII expression and phosphorylation in overloaded muscle to be significantly different than sham muscle on the same days.

CHAPTER 2: METHODS AND MATERIALS

Animals

All experimental procedures were approved by the East Carolina University Institutional Animal Care and Use Committee (IACUC), and are in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals. Male mice of the CD-1 strain with a starting weight of 35 to 40 grams were purchased from Charles River Laboratories (Raleigh-Durham, NC) and then housed in cages at 21-22°C with a 12 hr light/dark cycle. Rodent chow (ProLab RMH300, Lab Diet, Brentwood, MO) and water were available ad libitum.

Unilateral Denervation Surgery

Denervation is defined as the loss of nerve supply to a tissue in the body. In this study, a section of the sciatic nerve was removed from the hind leg of mice, resulting in atrophy of the denervated skeletal muscles (Shavlakadze et al., 2005). The following procedure for unilateral hindlimb denervation was adapted and modified from Shavlakadze et al. Mice were weighed then anesthetized with 2-3% isoflurane. The upper portion of the hindlimb was shaved and an incision made on the proximal thigh. A blunt dissection was performed on the quadriceps muscles to expose the sciatic nerve. A ~0.5 cm segment of the sciatic nerve bundle was removed, and thus, the lower leg no longer received nerve impulses. Removal of at least 0.5 cm of the nerve was sufficient to prevent any rejoining of the sciatic nerve. The incision was closed with surgical glue (Vetbond, 3M).

A sham operation was performed on the opposite leg of each mouse. The sham operation was performed exactly the same as the denervation surgery except that the sciatic nerve was not severed or removed. Mice were euthanized and the plantaris removed from the mice 1, 3, 7, 10,

and 14 days after surgery. The muscles were immediately frozen in liquid nitrogen, and then weighed.

Unilateral Synergist Ablation Surgery

Synergist ablation surgery requires the removal of one or more muscles from a synergist group. The remaining muscle of the synergist group is not removed and functional overload (hypertrophy) of the muscle is observed. The following procedure for synergist ablation has been adapted from those described by Thomson and Gordon (2006.)

Mice were weighed and anesthetized with 2-3% isoflurane solution. The fur on the back of the leg was removed with a shaver, and the skin sterilized with betadine solution. An incision was made on the posterior portion of one leg and the distal 60-70% of the gastrocnemius and soleus muscles removed, leaving the plantaris muscle intact. Removing only the distal two-thirds prevented any complications dealing with the knee capsule and its surrounding muscles and nerves. After removal of the gastrocnemius and soleus muscles, the incision was closed with sutures.

Critical to this surgery was the sham (control) operation performed on the opposite leg. This included creating an incision on the control leg and isolating the gastrocnemius and soleus but not removing these muscles. After surgery, the mice were monitored every day for signs of stress or infection until euthanasia. Euthanasia occurred 1, 3, 7 or 10 days after surgery and the plantaris muscles removed. Plantaris muscles were immediately frozen in liquid nitrogen, and then weighed.

Tissue Homogenization

Frozen muscles were processed for Western blot analyses as described by Witczak et al. (2006). Muscles were pulverized and then homogenized in bullet blender tubes with 1.4 mm diameter stainless steel beads and 200 µl of buffer solution containing 20 mM Tris-HCl, pH 7.5, 5 mM EDTA, 10 mM $Na_4P_2O_7.10H_2O$, 1% Nonidet P-40, 100 mM NaF, 10 µg ml^{-1} leupeptin, 10 µg ml^{-1} aprotinin and 2 mM Na_3VO_4, 3 mM benzamidine, and 1 mM phenylmethylsulfonyl fluoride (PMSF). The homogenate was rotated end over end for 1 hour at 4°C and then centrifuged at 13,000 x g for 30 min. The supernatant (lysate) was removed and stored at -80°C. Concentrations of the total protein content were determined by the Bradford method (Bradford, 1976) with BSA as a reference protein.

Western Blotting

Muscle lysates (20 µg total protein) were placed in 5 µl of 4x Laemmli's buffer and heated for 5 minutes at 95°C. The samples were loaded onto 1.5 mm, 15-well, 8% SDS-polyacrylamide gels and separated using a Mini-Protean Tetra cell electrophoresis apparatus (Bio-Rad) at 180 volts for 45-50 minutes (or until the dye runs off). The gels were put on nitrocellulose membranes and placed between two filter papers and two transfer sponges within a plastic transfer cassette. Proteins were then transferred onto the nitrocellulose membranes for immunodetection using a Mini Trans-Blot cell apparatus (Bio-Rad) at 100 volts for 1 hour. The membranes were placed in 20 ml of a blocking solution composed of 5% bovine serum albumin (BSA) dissolved in 1x Tris-buffered saline (1x TBS) + 0.1% Tween-20 for 1 hour. Primary antibodies that detect total CaMKIIβ_M, γ, and δ (Santa Cruz Biotechnology, catalog#sc-9035) or phospho-CaMKII (Thr 286/287) (Cell Signaling Technology, catalog#3361) were utilized to

detect total and phosphorylated CaMKII, respectively. For total CaMKII, a 1:4000 dilution of the primary antibody was made in 20 ml of 5% BSA in 1x TBS + 0.1% Tween + 0.01% NaN_3. For the phospho-CaMKII primary antibody, a 1:2000 dilution was made in 20 ml of 5% BSA in 1x TBS + 0.1% Tween + 0.01% NaN_3. The membranes were incubated with rocking overnight at 4 °C. Membranes were washed 5 times in 20 ml each of 1x TBS + 0.1% Tween to remove any unbound primary antibody and incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody for one hour at room temperature. For total CaMKII detection, a 1:5000 dilution of anti-mouse (IgG)-HRP (Millipore Corp, catalog#12-349) was made in 20 ml of 5% BSA in1x TBS + 0.1% Tween. For phospho-CaMKII detection, a 1:2000 dilution of anti-rabbit (IgG)-HRP (ThermoScientific, catalog#31460) was made. The membrane was washed 5 times with 20 ml of 1x TBS + 0.1% Tween.

The membranes were dried by dabbing the sides against paper towels and then placed on plastic wrap. Western Lightning Plus-ECL reagents (Perkin Elmer, catalog#NEL105001) were combined into a 50 ml BD Falcon Tube (1:1 ratio) and 1 ml of the solution was pipetted onto each membrane. The luminescence intensity of the bands was quantified using a ChemiDoc XRS+ imaging system and Image Lab™ software (Bio Rad). Band intensity was normalized to the intensity of internal control samples as described below. Within a set of Western blots, every blot contained the exact same muscle sample, which was defined as the internal control. The intensity of the internal control on one gel was compared to the internal control on the other gels, and utilized to calculate a normalization factor. For example, if the intensity of the internal control sample on gel 2 was 1.5x greater than the intensity of the internal control sample on gel 1, then all of the samples on gel 2 would be divided by 1.5 to normalize the apparent gel-to-gel

variability in the western blotting procedures. The normalized intensity values were then grouped based on treatment, and the mean and standard error of the mean calculated.

Statistical Analysis

The data are presented as the mean ± standard error of the mean. Statistical significance was defined as $P < 0.05$ and determined by paired t-tests or by two-way analysis of variance (ANOVA) and Student-Newman-Keuls post hoc analysis. The number of mice utilized to determine significance is indicated in the figure legend.

CHAPTER 3: RESULTS

Denervation Surgery

Mouse Body Weights

To determine the effects of skeletal muscle atrophy on CaMKII (Thr286/287) phosphorylation and CaMKII protein expression, mice underwent unilateral denervation of the hind limb via sciatic nerve resection. Mice were weighed just prior to surgery and then every day until euthanasia. All 30 mice that underwent sciatic denervation surgery were euthanized on the same day. As shown in Table 1, none of the mice that underwent denervation surgery exhibited a significant decrease in body weight from the time of surgery to the time of euthanasia.

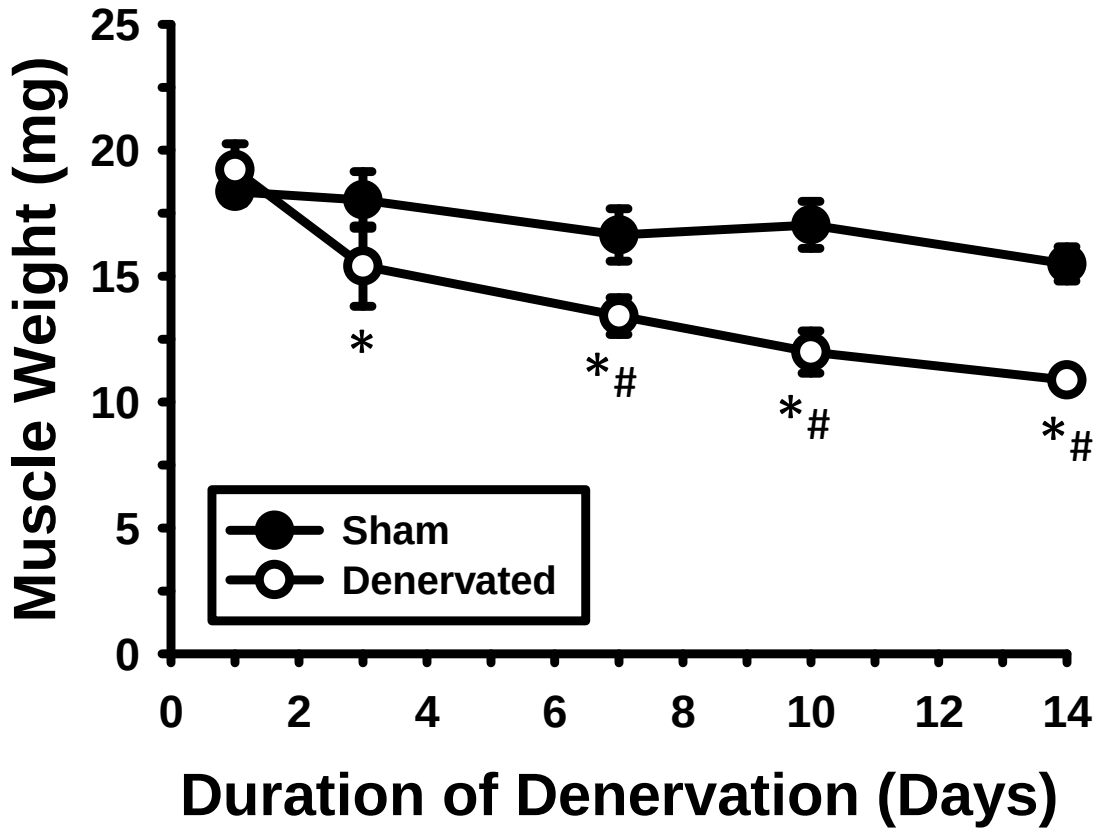
Duration of Denervation	Number of Mice	Body Weight on Surgery Day(g)	Body Weight on Euthanasia Day (g)	P-Value
1 Day	6	33.0 ± 0.3	32.9 ± 0.7	p= 0.566
3 Days	6	35.2 ± 1.1	35.0 ± 1.2	p= 0.067
7 Days	6	31.8 ± 0.7	33.1 ± 0.6	p< 0.001
10 Days	6	30.8 ± 0.6	32.8 ± 0.8	p< 0.001
14 Days	6	29.1 ± 0.3	33.1 ± 0.9	p= 0.003

Table 1: Body Weights on Surgery Day and the Day of Euthanasia for Mice that Underwent Denervation Surgery. Data are presented as the mean ± standard error of the mean. Statistical significance between body weights on the day of surgery vs. the day of euthanasia was assessed by paired t-test.

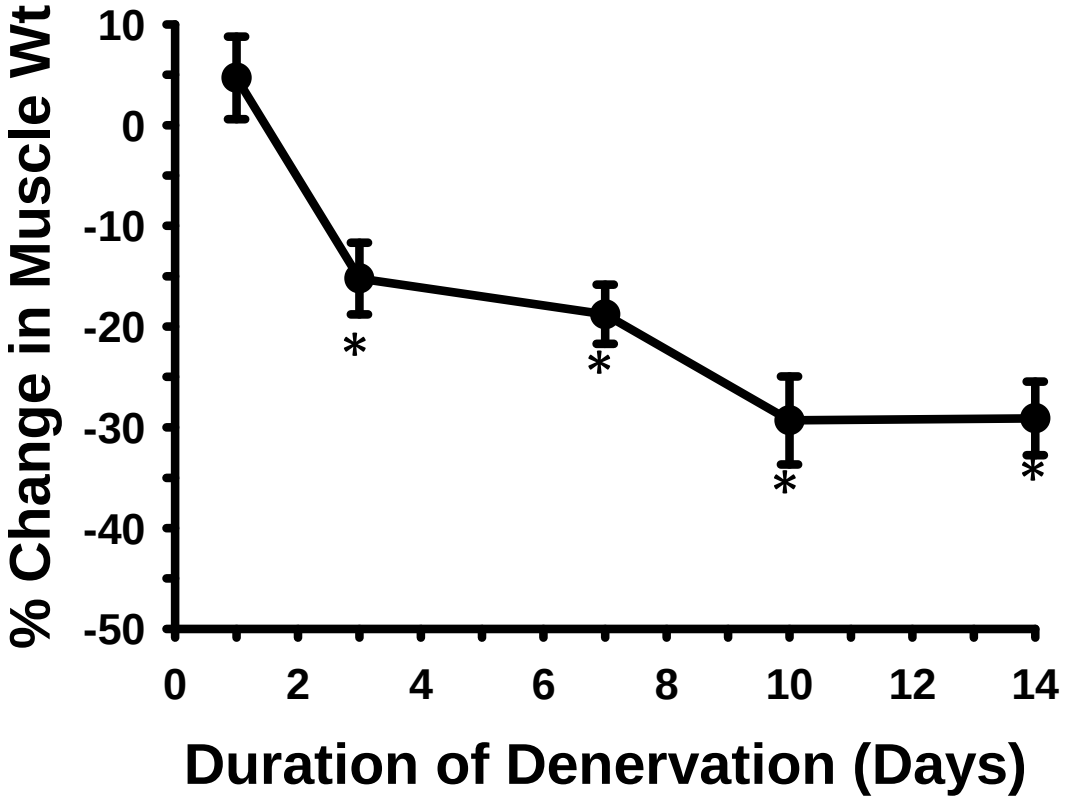
Mouse Plantaris Muscle Weights

Plantaris muscles were collected 1, 3, 7, 10 or 14 days after the denervation surgery, frozen in liquid nitrogen and then weighed. The effect(s) of muscle denervation were determined by examination of raw muscle weights as well as the calculation of the percent change in muscle weight from the denervated muscle to the sham muscle within the same animal. At 1 day and 3 days after the denervation surgery, there was no significant muscle atrophy observed in the denervated plantaris muscles compared to the sham-operated control muscles of the same day (Fig 9). Denervated plantaris muscles from the 7 day, 10 day and 14 day mice all showed a significant decrease in weight when compared to their respective sham muscles (Fig 9). None of the sham plantaris muscle weights were statistically different from each other (Fig 9).

Consistent with the data for muscle weights, the average percent change in muscle weights following 1 day of denervation was not different than 0% demonstrating no muscle atrophy at this time point. Following 3, 7, 10 and 14 days of denervation, the average percent change in plantaris muscle weight was significantly decreased compared to day 1 (Fig 10).



<u>Figure 9</u>: Effect of Denervation on Mouse Plantaris Muscle Weights. Data are presented as the mean ± standard error of the mean. Statistical significance was assessed by Two-way ANOVA and Student-Newman-Keuls post-hoc analysis, and defined as $p < 0.05$ with '*' vs. Day 1 and '#' vs. sham muscles from the same day. For all experimental groups, N= 6 muscles.



<u>Figure 10</u>: Percent Change in Mouse Plantaris Muscle Weights Following Denervation. Data are presented as the mean ± standard error of the mean. Statistical significance was assessed by One-way ANOVA and Student-Newman-Keuls post-hoc analysis, and defined as $p < 0.05$, with '*' vs. Day 1. For all experimental groups, N= 6 muscles.

Western Blot Analyses for CaMKII Phosphorylation and Expression

The frozen plantaris muscles were processed for Western blot analyses to assess CaMKII (Thr 286/287) phosphorylation and protein expression. Both the phospho-CaMKII antibody and the total CaMKII antibody detect all known isoforms of CaMKII found in skeletal muscle (i.e. CaMKIIβ_M, γ, and δ), and the initial intent of this study was to quantify all of the CaMKII isoforms together. However, due to noticeable differences in the intensity of the bands for the phosphorylation and protein expression blots, the three separate isoforms of CaMKII in skeletal muscle were quantified separately.

Based on amino acid sequence and composition, the predicted molecular weights of the different CaMKII isoforms are ~72 kDa for the CaMKIIβ_M isoform, ~62 kDa for the CaMKIIγ isoform, and ~57 kDa for the CaMKIIδ isoform. Thus, for clarity in this thesis, the bands detected by the phospho- and total CaMKII antibodies will be referred to as CaMKIIβ_M, CaMKIIγ, and CaMKIIδ based on molecular weight.

CaMKII Phosphorylation. Figures 11 through 13 contain representative blots and the quantification of CaMKIIβ_M, CaMKIIγ and CaMKIIδ (Thr286/287) phosphorylation. There were no significant differences in CaMKIIβ_M phosphorylation induced by the denervation surgery at any of the time points examined (Fig 11). The phosphorylation of CaMKIIγ was significantly decreased in the denervated muscles by days 7, 10 and 14 compared to day 3 (Fig 12). Similar to the results obtained for CaMKIIβ_M phosphorylation, there were no significant differences in CaMKIIδ (Thr286/287) phosphorylation induced by denervation at any of the time points examined (Fig 13). Phosphorylation of the sham plantaris muscles for all three isoforms was not statistically different at any time following denervation surgery (Fig 11,12,13).

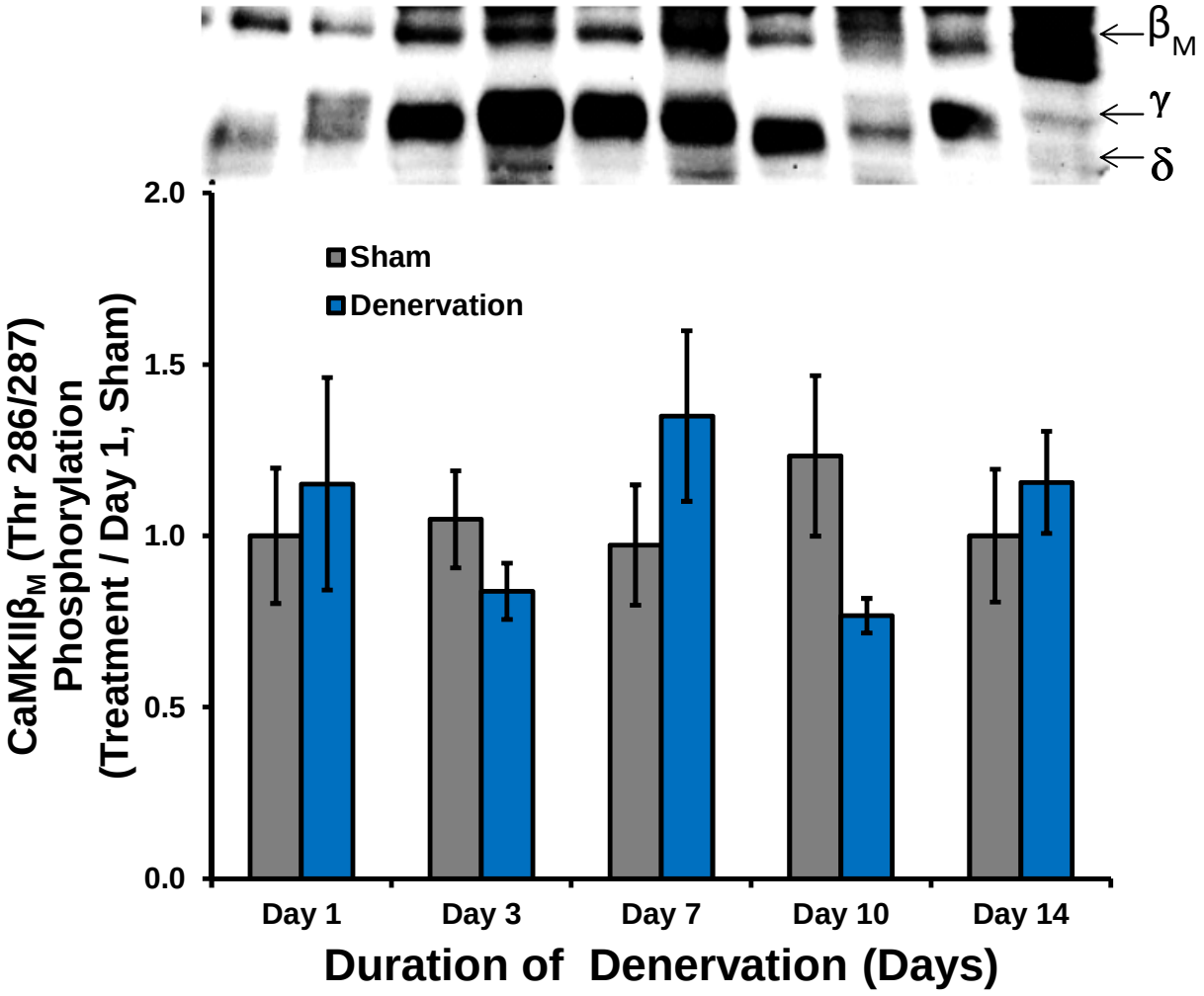
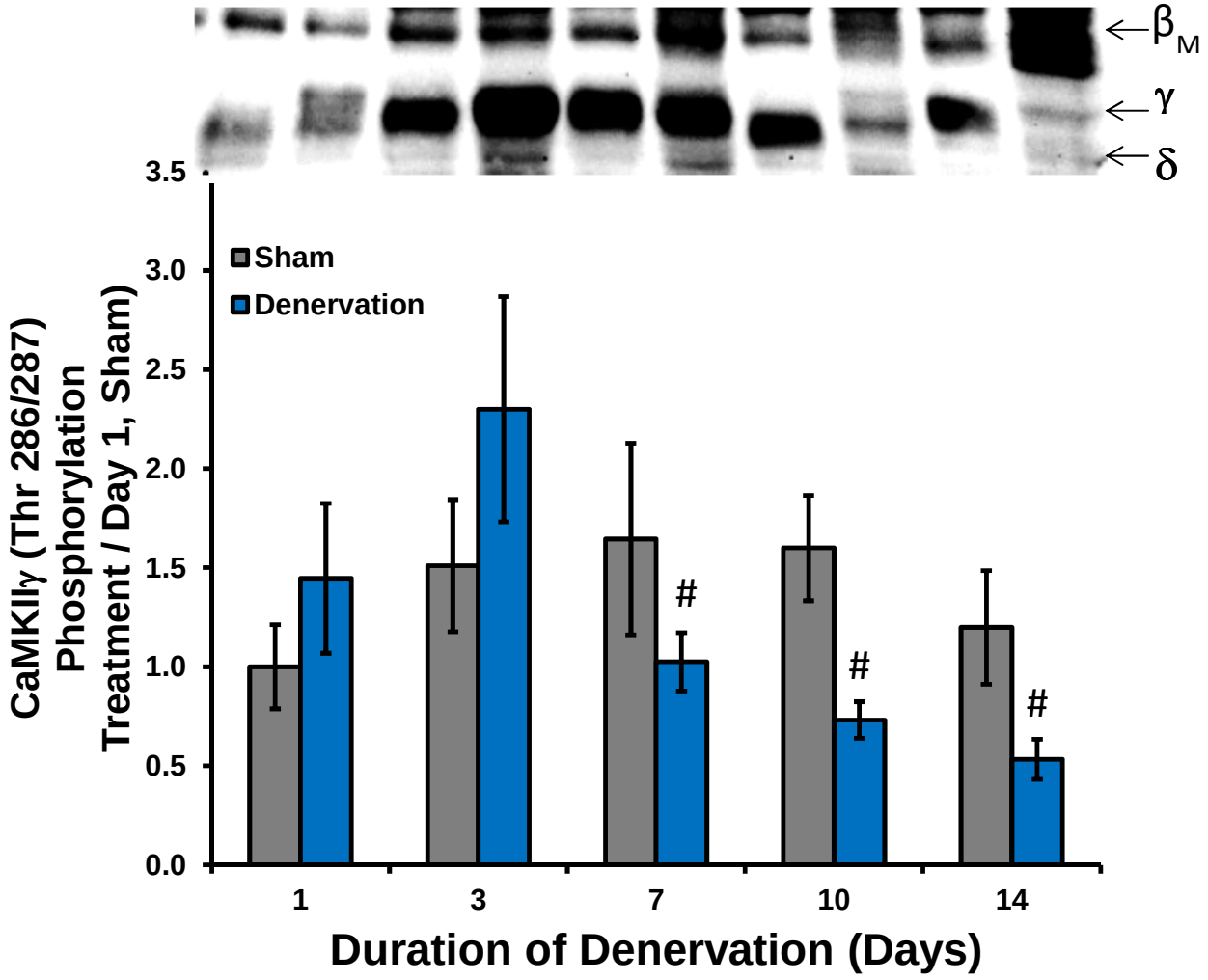
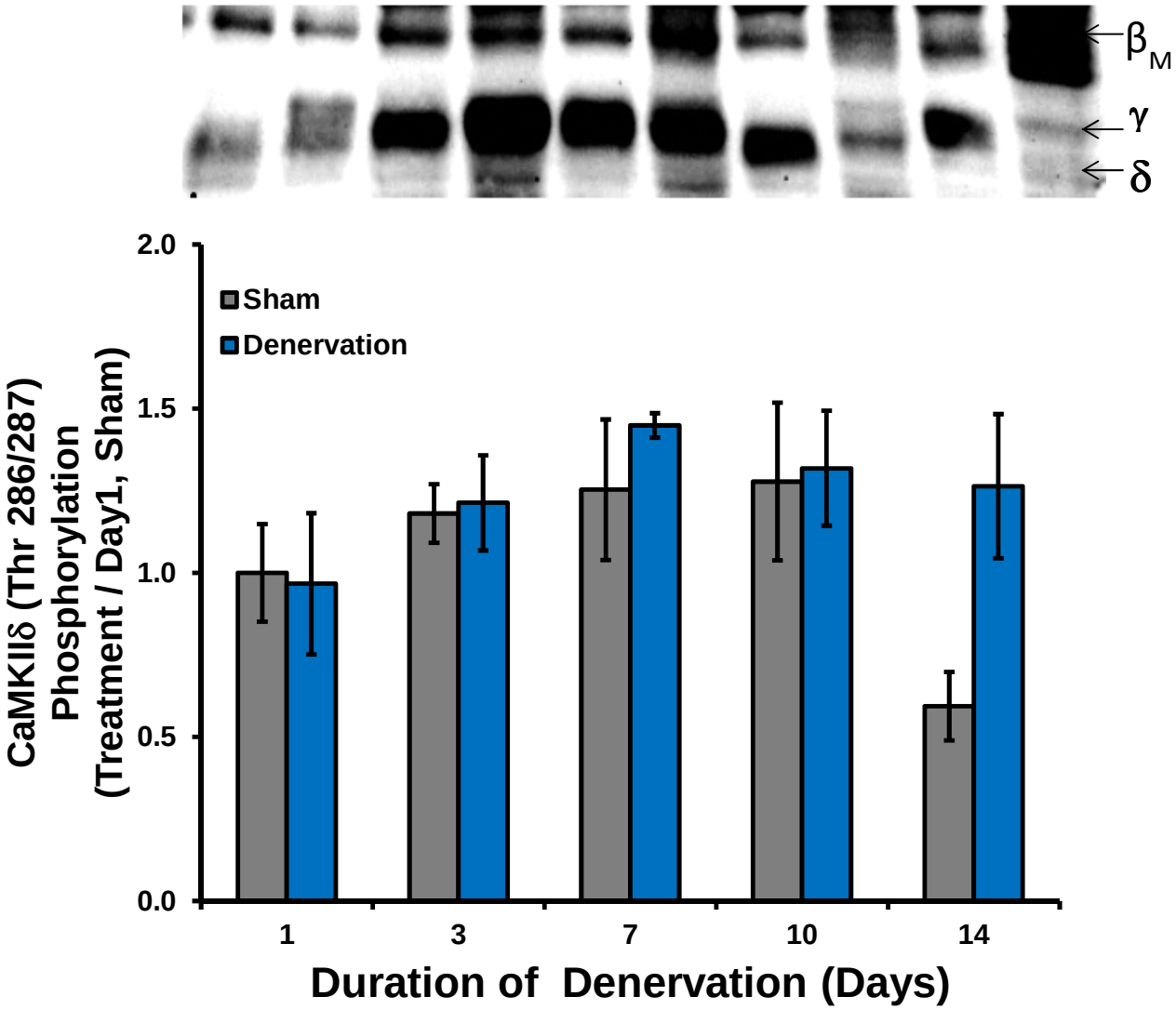


Figure 11: Effect of Denervation on CaMKIIβ_M (Thr286/287) Phosphorylation in Mouse Plantaris Muscles. Data are presented as the mean ± standard error of the mean. Statistical significance was assessed by Two-way ANOVA and Student-Newman-Keuls post-hoc analysis, and defined as $p < 0.05$. All other groups, N=5-6 muscles/group.



<u>Figure 12</u>: Effect of Denervation on CaMKIIγ (Thr286/287) Phosphorylation in Mouse Plantaris Muscles. Data are presented as the mean ± standard error of the mean. Statistical significance was assessed by Two-way ANOVA and Student-Newman-Keuls post-hoc analysis, and defined as $p < 0.05$, with '#' vs. Day 3. N=4-6 muscles/group.



<u>Figure 13</u>: Effect of Denervation on CaMKIIδ (Thr286/287) Phosphorylation in Mouse Plantaris Muscles. Data are presented as the mean ± standard error of the mean. Statistical significance was assessed by Two-way ANOVA and Student-Newman-Keuls post-hoc analysis, and defined as $p < 0.05$. N=5-6 muscles.

CaMKII Protein Expression. Figures 14 through 16 show representative blots and the quantification of band intensity for CaMKIIβ_M, CaMKIIγ and CaMKIIδ protein expression from the plantaris muscles of denervated and sham mouse legs. The denervated plantaris muscles on days 3, 7, 10 and 14 have significantly lower CaMKIIβ_M expression when compared to their sham plantaris muscles (Fig 14). In contrast, following 10 and 14 days of denervation, there was a significant increase in CaMKIIγ protein expression compared to the sham-operated control muscles as well as denervated muscles on days 3 and 7 (Fig 15). Figure 16 shows a significant increase (~200%) in CaMKIIδ expression in the denervated plantaris muscles on day 10 and day 14 when compared to their sham muscles. In addition, CaMKIIδ expression in the denervated muscles from 10 day and 14 day mice was also significantly higher than CaMKIIδ expression at 1, 3, and 7 days after denervation (Fig 16). CaMKIIβ_M, γ, and δ protein expression was not statistically different in any of the sham muscles at any time point following denervation surgery (Fig 14,15,16).

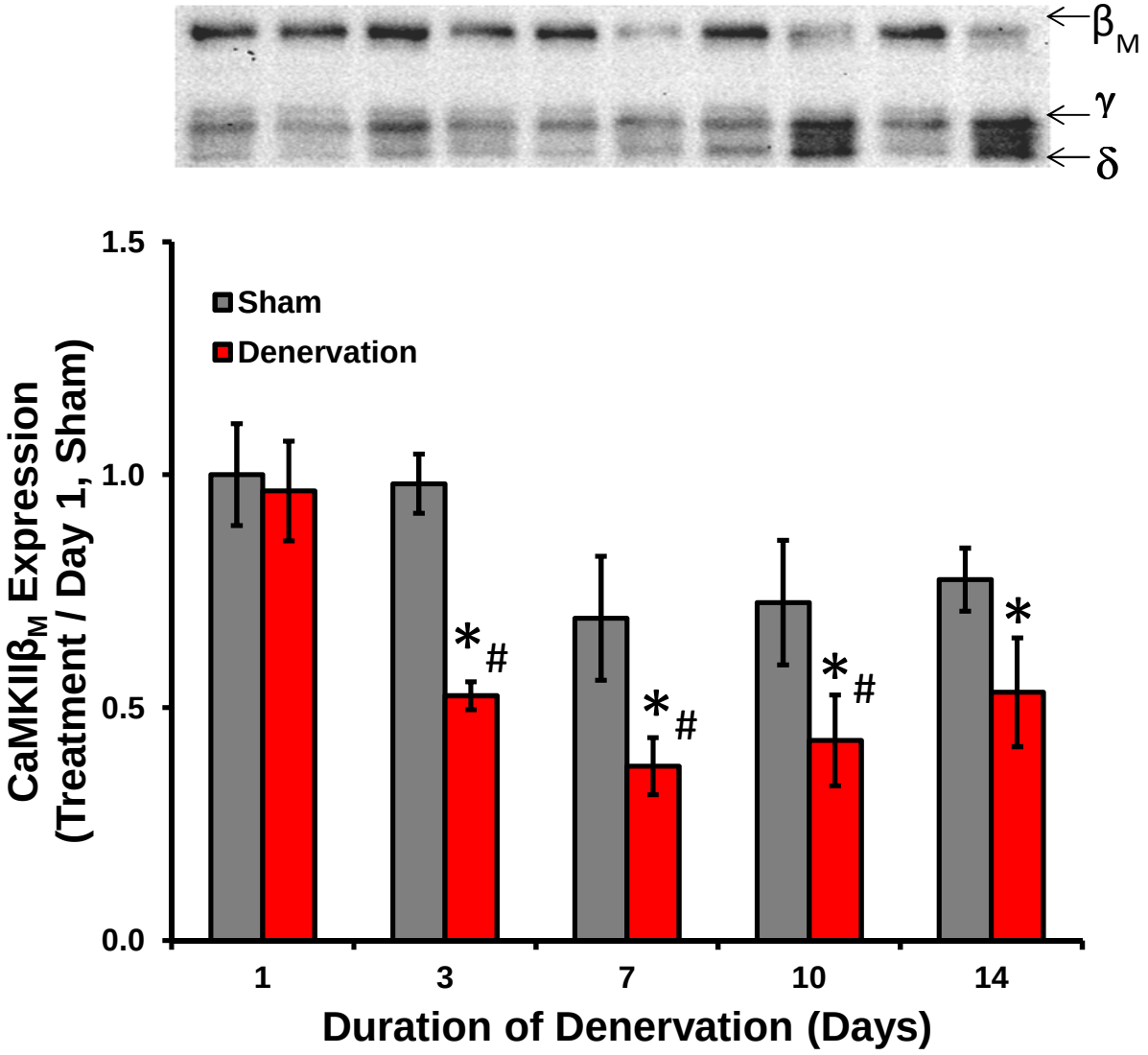


Figure 14: Effect of Denervation on CaMKIIβ_M Protein Expression in Mouse Plantaris Muscles. Data are presented as the mean ± standard error of the mean. Statistical significance was assessed by Two-way ANOVA and Student-Newman-Keuls post-hoc analysis, and defined as $p < 0.05$, with '#' vs. Day 1 and '*' vs. sham muscles from the same day. N=5-6 muscles/group.

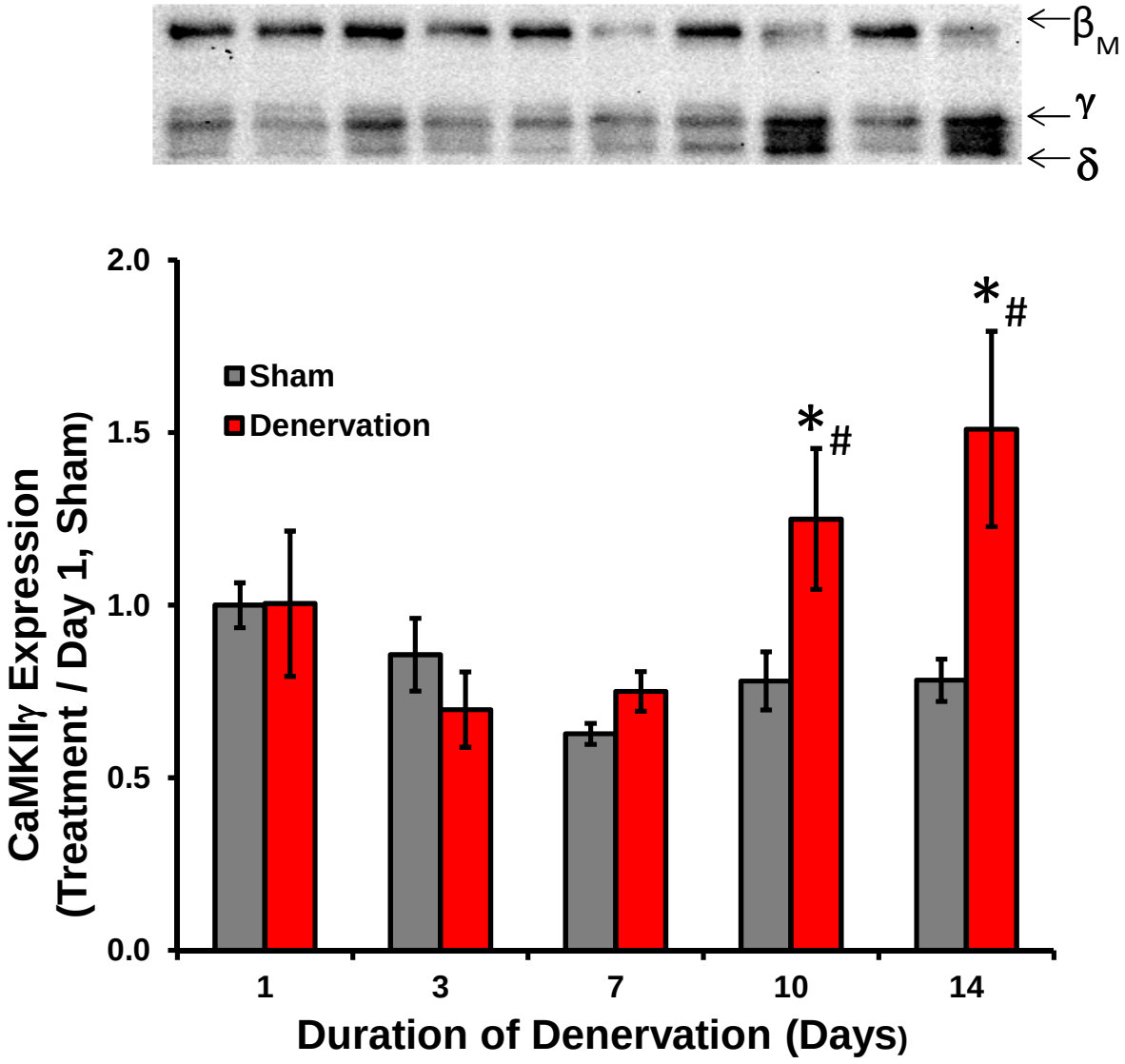


Figure 15: Effect of Denervation on CaMKIIγ Protein Expression in Mouse Plantaris Muscles. Data are presented as the mean ± standard error of the mean. Statistical significance was assessed by Two-way ANOVA and Student-Newman-Keuls post-hoc analysis, and defined as $p < 0.05$, with '#' vs. Day 3 and Day 7 and '*' vs. sham muscles from the same day. N=5-6 muscles/group.

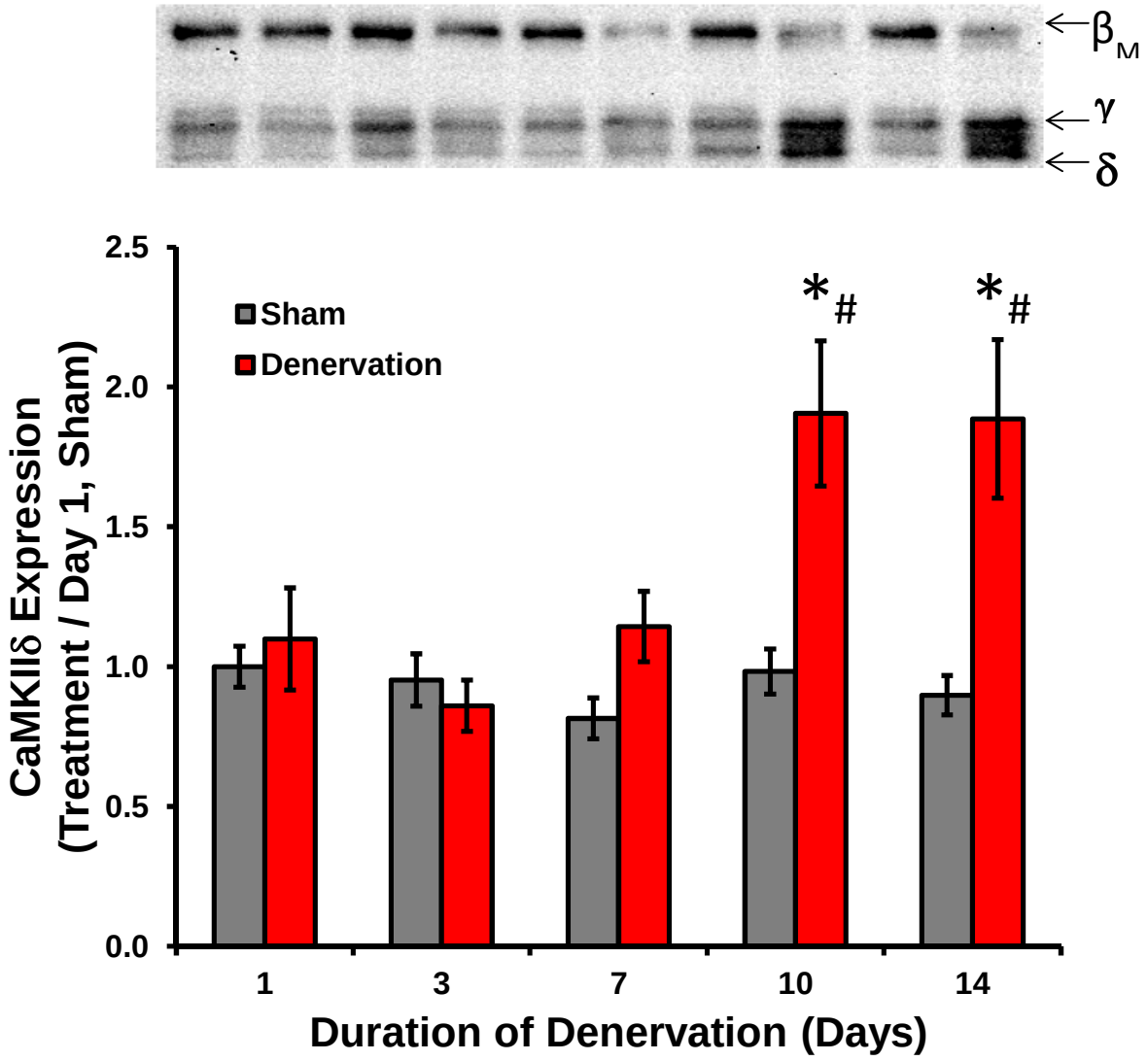
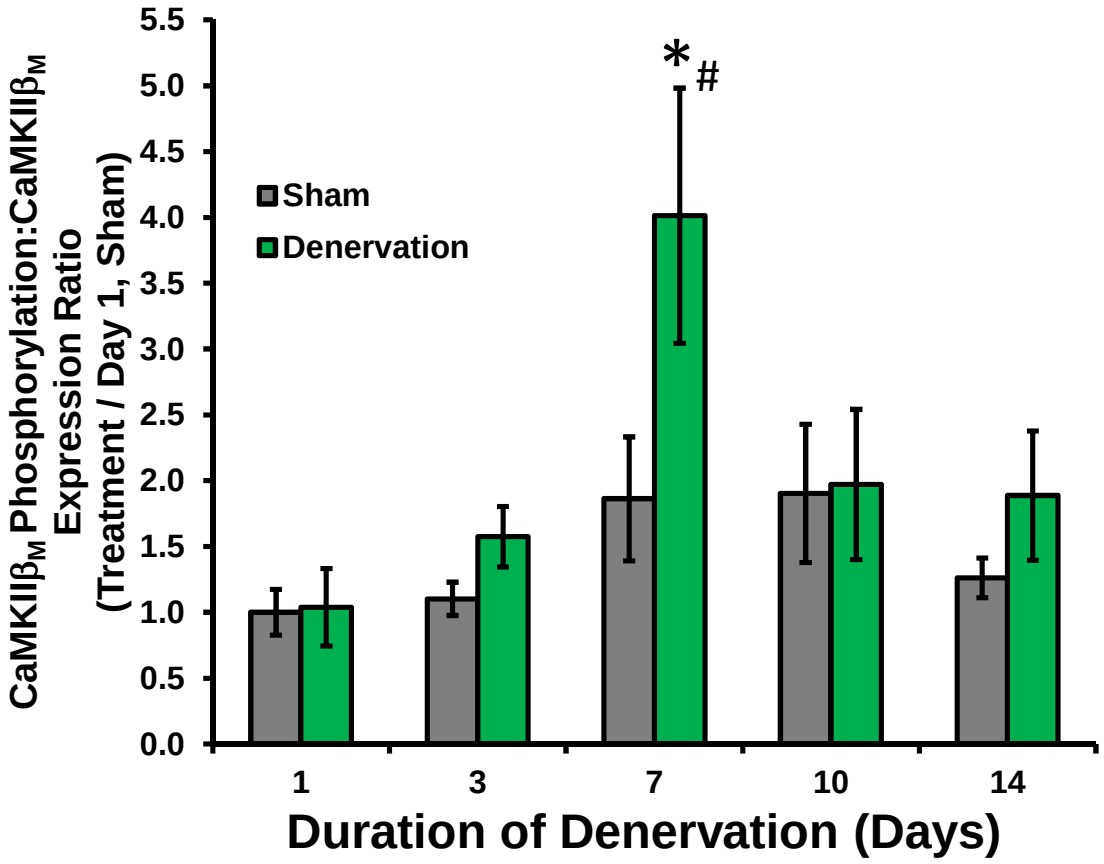


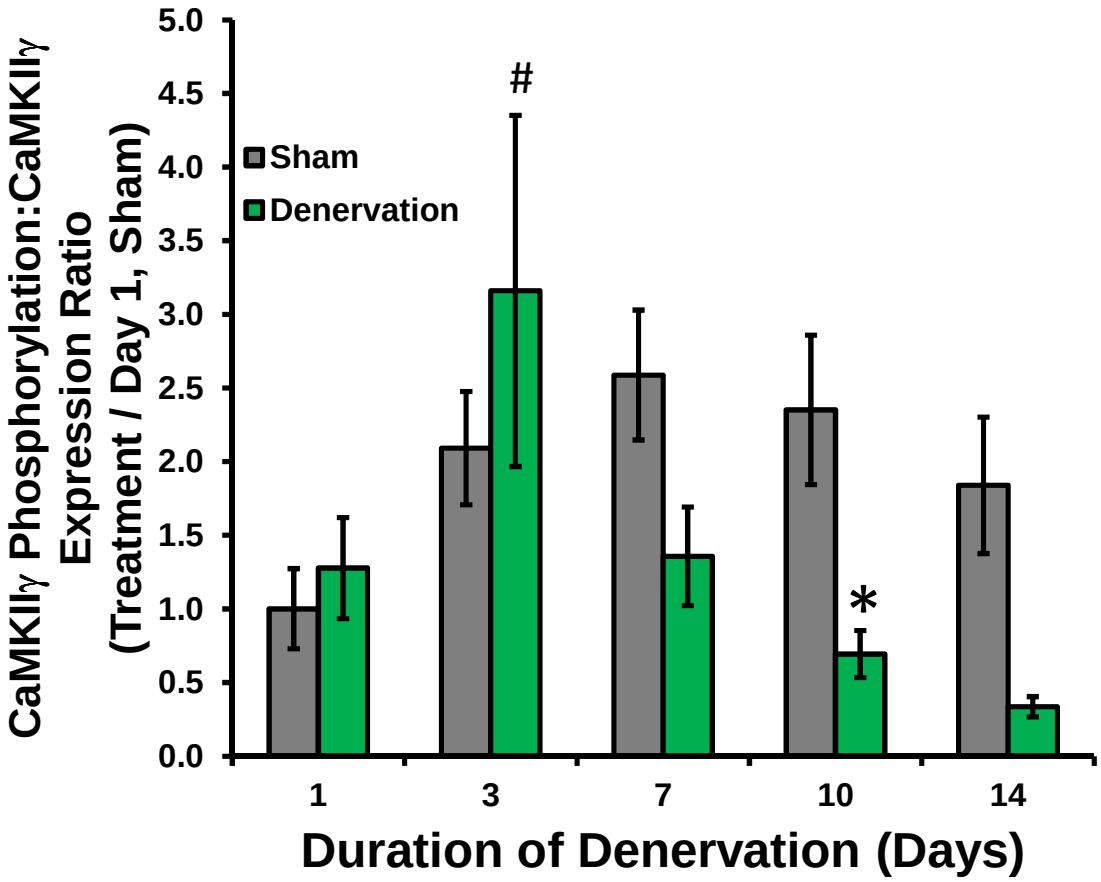
Figure 16: Effect of Denervation on CaMKIIδ Protein Expression in Plantaris Muscles. Data are presented as the mean ± standard error of the mean. Statistical significance was assessed by Two-way ANOVA and Student-Newman-Keuls post-hoc analysis, and defined as $p < 0.05$, with '#' vs. Day 1, Day 3 and Day 7 and '*' vs. sham muscles from the same day. N=5-6 muscles/group.

Ratio of Phosphorylated CaMKII to Total CaMKII. The ratio of phosphorylated CaMKII to CaMKII protein expression (phospho:total) was determined for each isoform in order to gain a better perspective on how expression and phosphorylation together effect the overall activation of CaMKII throughout the denervation time course.

Figures 17 through 19 show the phospho:total ratios of each isoform ($CaMKII\beta_{M,}$ CaMKIIγ and CaMKIIδ) from the plantaris muscles of denervated and sham mouse legs. The ratio of phosphorylated $CaMKII\beta_M$ to $CaMKII\beta_M$ protein expression was significantly increased in the denervated plantaris muscles at day 7, but unchanged at any other time point (Fig 17). There was no change in the $CaMKII\beta_M$ phospho to total ratio in the sham-operated muscles at any time point (Fig 17). The ratio of phosphorylated CaMKIIγ to total CaMKIIγ on day 10 was significantly lower than the respective sham ratio (Fig 18). The denervated muscles on day 3 contain a significantly higher CaMKIIγ phospho:total ratio when compared to the other denervated phospho:total ratios on day 1, 7, 10 and 14 (Fig 18). For CaMKIIδ, there were no significant differences in the denervation phospho:total ratios compared to sham ratios at any day during the time course except on day 10 (Fig 19). Also, the sham-operated muscles on day 14 contain a significantly lower CaMKIIδ phospho:total ratio compared to day 7 sham-operated control muscles (Fig 19).



<u>Figure 17</u>: Effect of Denervation on the Ratio of CaMKIIβ$_M$ (Thr286/287) Phosphorylation to CaMKIIβ$_M$ Protein Expression in Mouse Plantaris Muscles. Data are presented as the mean ± standard error of the mean. Statistical significance was assessed by Two-way ANOVA and Student-Newman-Keuls post-hoc analysis, and defined as $p < 0.05$, with '#' vs. Day 1, Day 3 and Day 10 and Day 14 and '*' vs. sham muscles from the same day. N=5-6 muscles/group.



<u>Figure 18</u>: Effect of Denervation on the Ratio of CaMKIIγ (Thr286/287) Phosphorylation to CaMKIIγ Protein Expression in Mouse Plantaris Muscles. Data are presented as the mean ± standard error of the mean. Statistical significance was assessed by Two-way ANOVA and Student-Newman-Keuls post-hoc analysis, and defined as $p < 0.05$, with '#' vs. Day 1, Day 7, Day 10 and Day 14 and '*' vs. sham muscles from the same day. N=5-6 muscles/group.

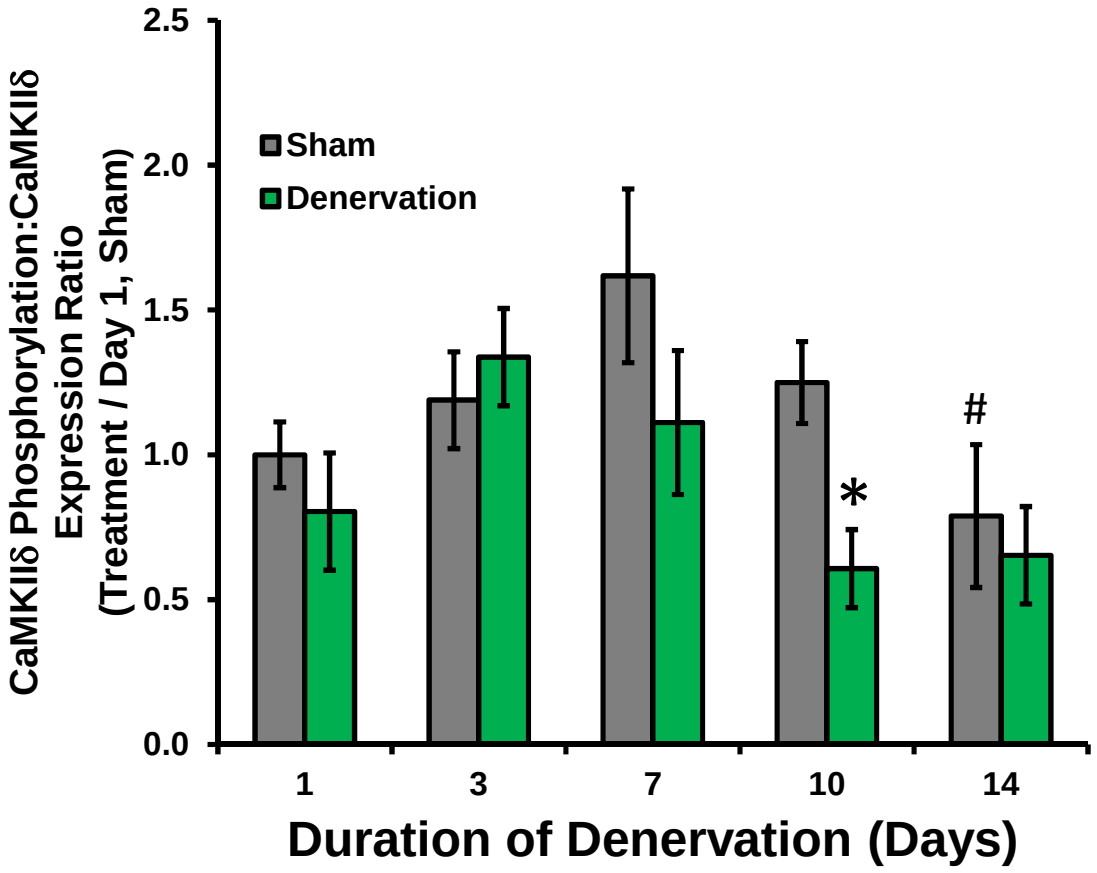


Figure 19: Effect of Denervation on the Ratio of CaMKIIδ (Thr286/287) Phosphorylation to CaMKIIδ Protein Expression in Mouse Plantaris Muscles. Data are presented as the mean ± standard error of the mean. Statistical significance was assessed by Two-way ANOVA and Student-Newman-Keuls post-hoc analysis, and defined as $p < 0.05$, with '#' vs. Day 7 sham and '*' vs. sham muscles from the same day. N=5-6 muscles/group.

Synergist Ablation Surgery

Mouse Body Weights

To determine the effects of skeletal muscle hypertrophy on CaMKII (Thr286/287) phosphorylation and CaMKII protein expression, mice underwent unilateral synergist ablation surgery of the hind limb via removal of the gastrocnemius and soleus muscles. This surgery induces hypertrophy of the remaining synergist muscle, i.e. the plantaris muscle. Mice were weighed just prior to surgery and then every day until euthanasia. All 31 mice that underwent synergist ablation surgery were euthanized on the same day. As shown in Table 2, none of the mice that underwent synergist ablation surgery exhibited a significant decrease in body weight from the time of surgery to the time of euthanasia.

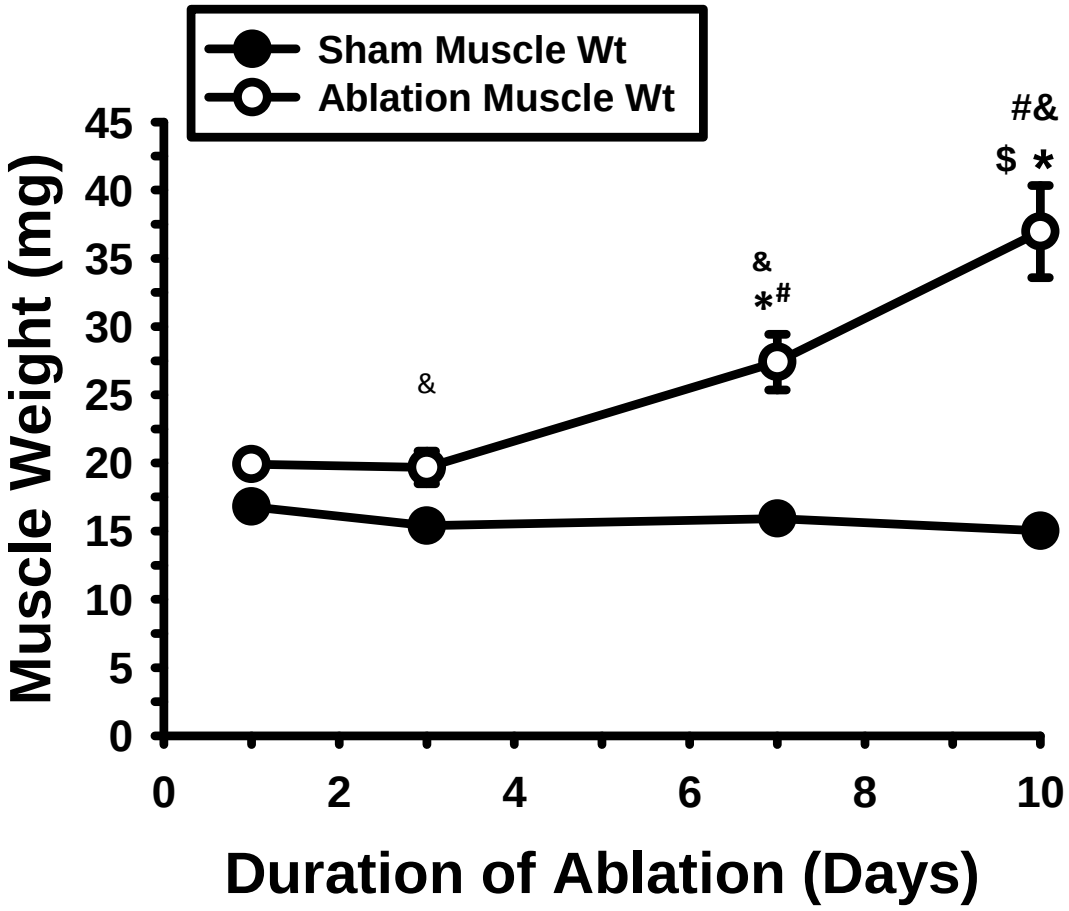
Duration of Ablation	Number of Mice	Body Weight on Surgery Day(g)	Body Weight on Euthanasia Day (g)	P-Value
1 Day	8	32.8±0.4	32.2±0.5	p=0.027
3 Days	8	32.4±0.5	31.8±0.5	p=0.011
7 Days	8	28.0±0.6	30.6±0.5	p<0.001
10 Days	7	25.8±0.5	30.4±0.9	p<0.001

Table 2: Mouse Body Weights on Surgery Day and the Day of Euthanasia for Mice that Underwent Synergist Ablation Surgery. Data are presented as the mean ± standard error of the mean. Statistical significance between body weights on the day of surgery vs. the day of euthanasia was assessed by paired t-test.

Mouse Plantaris Muscle Weights

Plantaris muscles were collected 1, 3, 7 or 10 days after the synergist ablation surgery, frozen in liquid nitrogen and then weighed. The effect(s) of functional overload on muscle mass were determined by examination of raw muscle weights as well as the calculation of percent change in muscle weight from the overloaded muscle to the sham-operated muscle within the same animal. At 1 day following the ablation surgery, plantaris muscles showed no significant change in weight in comparison to their sham muscles (Fig 20). The plantaris muscles from the 3 day, 7 day and 10 day ablated legs exhibited significant increase in weight when compared to their respective sham-operated plantaris muscles (Fig 20). None of the sham plantaris muscle weights were statistically different from each other (Fig. 20).

Plantaris muscles from the 1 day and 3 day ablated legs showed a 20-25% increase in weight compared the sham muscles from the same mice, and were not statistically different from each other (Fig 21). Following 7 and 10 days of overload, the percent change in plantaris muscle weights increased 75% and 150%, respectively (Fig 21).



<u>Figure 20</u>: Effect of Synergist Ablation on Mouse Plantaris Muscle Weights. Data are presented as the mean ± standard error of the mean. Statistical significance was assessed by Two-way ANOVA and Student-Newman-Keuls post-hoc analysis, and defined as $p< 0.05$, with '*' vs. Day 1, '#' vs. Day 3, '$' vs. Day 7 and '&' vs. sham muscles from the same day. N= 7-8 muscles/groups.

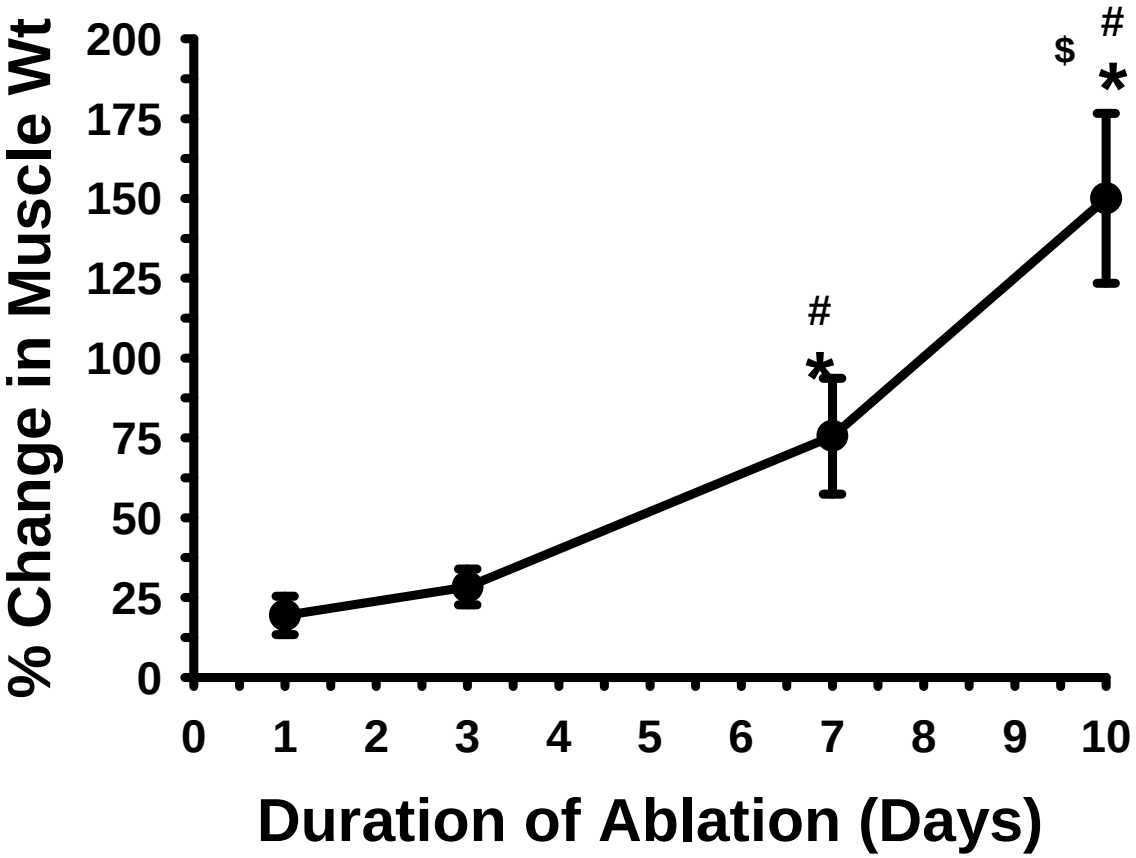


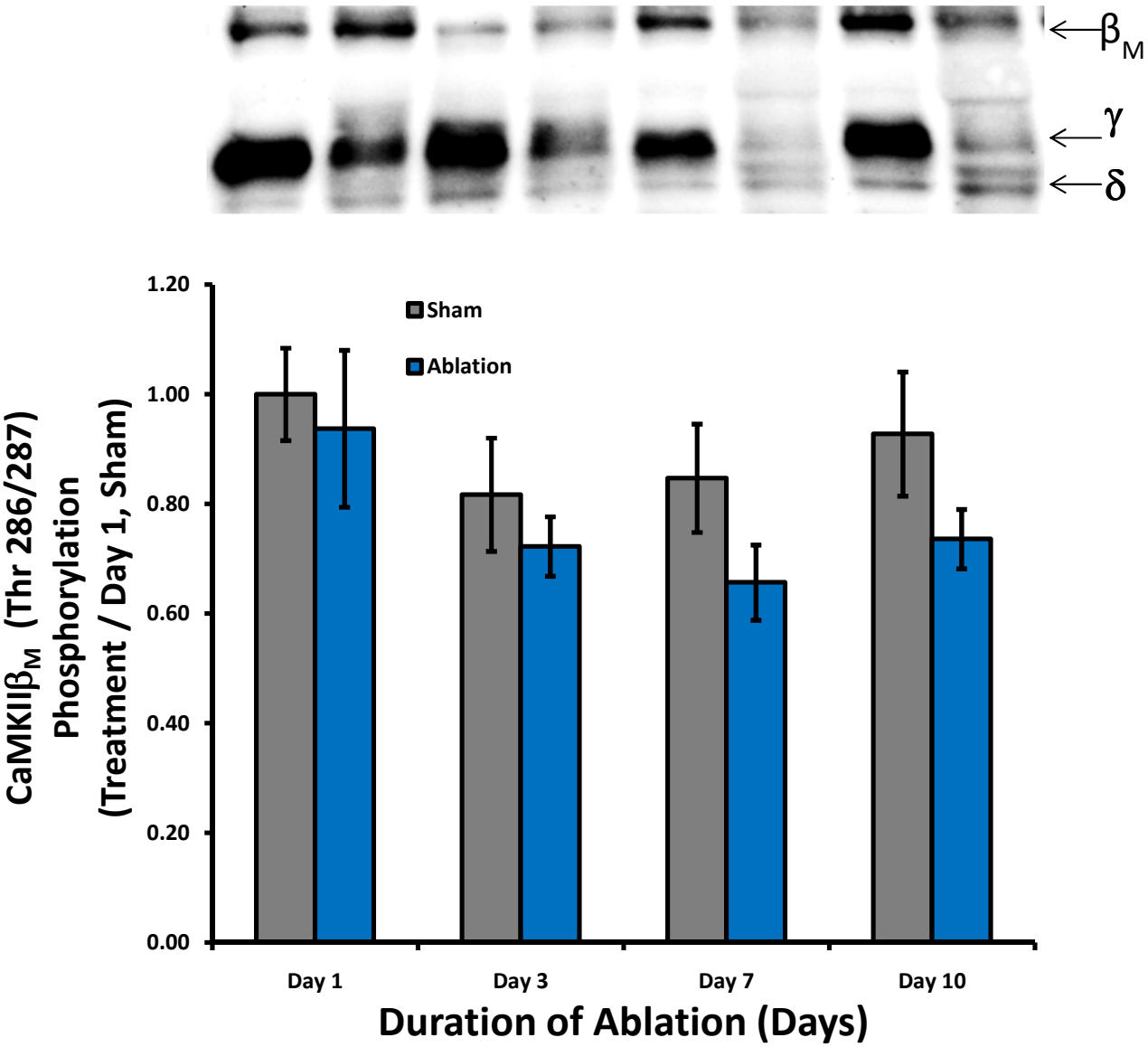
Figure 21: Percent Change in Experimental Plantaris Muscle Weights Following Synergist Ablation. Data are presented as the mean ± standard error of the mean. Statistical significance was assessed by One-way ANOVA and Student-Newman-Keuls post-hoc analysis, and defined as $p < 0.05$, with '*' vs. Day 1, '#' vs. Day 3 and '$' vs. Day 7. N= 7-8 muscles/group.

Western Blot Analyses for CaMKII Phosphorylation and Expression

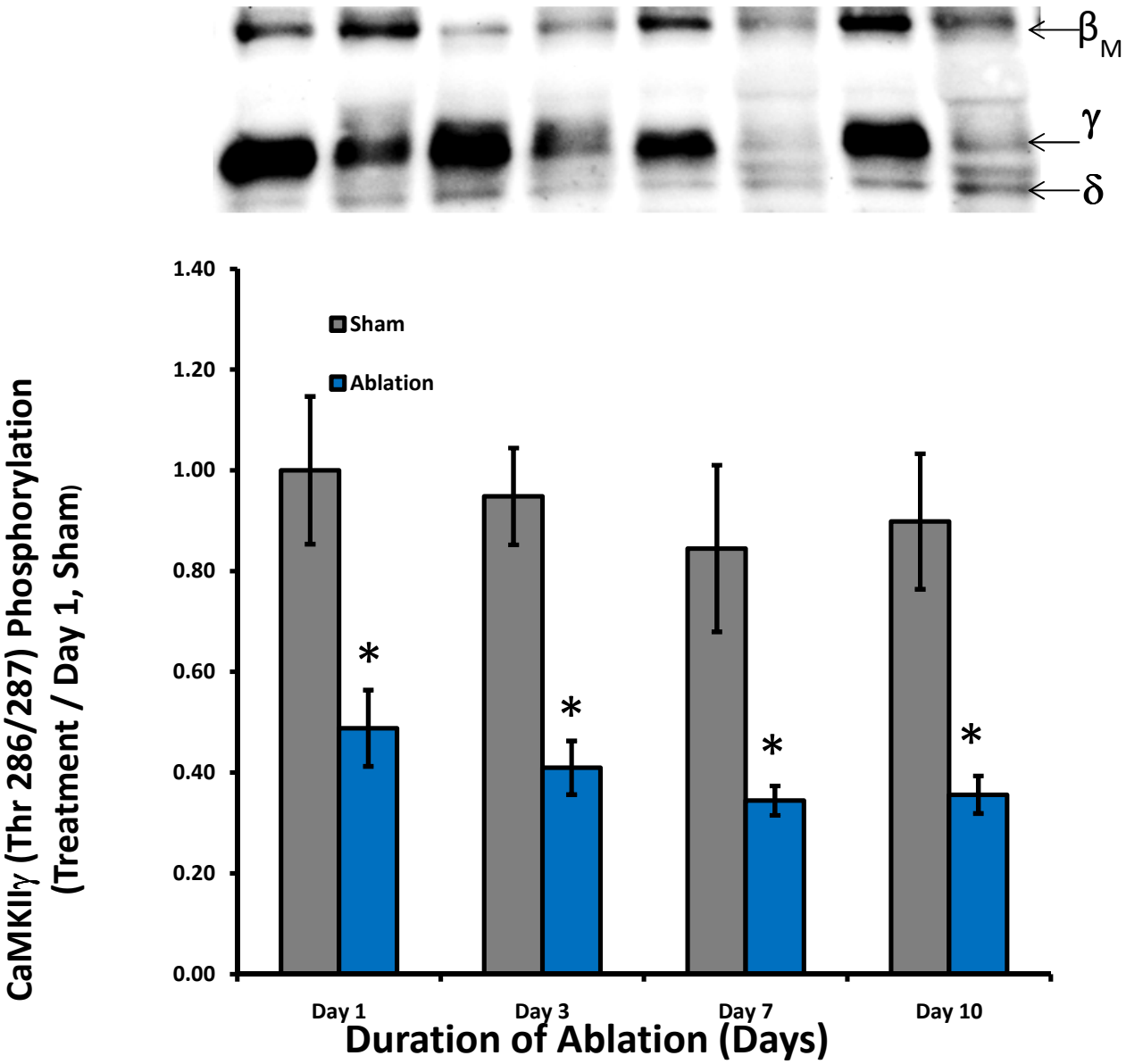
The frozen plantaris muscles were processed for Western blot analyses to determine CaMKII (Thr 286/287) phosphorylation and total CaMKII protein expression. Both the phospho-CaMKII antibody and the total CaMKII antibody detect all known isoforms of CaMKII found in skeletal muscle (i.e. CaMKIIβ_M, γ, and δ), and the initial intent of this study was to quantify all of the CaMKII isoforms together. However, due to noticeable differences in the intensity of the bands for the phosphorylation and protein expression blots, the three separate isoforms of CaMKII in skeletal muscle were quantified separately.

As previously mentioned in the "Denervation Surgery" section of the Results, the predicted molecular weights of the different CaMKII isoforms are ~72 kDa for the CaMKIIβ_M isoform, ~62 kDa for the CaMKIIγ isoform, and ~57 kDa for the CaMKIIδ isoform. Thus, the bands detected by the phospho- and total CaMKII antibodies will be referred to as CaMKIIβ_M, CaMKIIγ, and CaMKIIδ.

CaMKII phosphorylation. Figures 22 through 24 contain representative blots and the quantification of CaMKIIβ_M, CaMKIIγ and CaMKIIδ (Thr286/287) phosphorylation from overloaded and sham plantaris muscles. CaMKIIβ_M phosphorylation was not significantly altered by the synergist ablation surgery at any of the time points examined (Fig 22). In Figure 23, the overloaded plantaris muscles at 1, 3, 7 and 10 days after synergist ablation surgery all contained significantly lower CaMKIIγ phosphorylation compared to the sham muscles on the same days. Consistent with the results for CaMKIIβ_M phosphorylation, there were no significant alterations in CaMKIIδ phosphorylation induced by the ablation surgery at any of the time points (Fig 24).



<u>Figure 22</u>: Effect of Synergist Ablation on CaMKIIβ_M (Thr286/287) Phosphorylation in Mouse Plantaris Muscles. Data are presented as the mean ± standard error of the mean. Statistical significance was assessed by Two-way ANOVA and Student-Newman-Keuls post-hoc analysis, and defined as $p < 0.05$. N=6-8 muscles/group.



<u>Figure 23</u>: Effect of Synergist Ablation on CaMKIIγ Phosphorylation in Mouse Plantaris Muscles. Data are presented as the mean ± standard error of the mean. Statistical significance was assessed by Two-way ANOVA and Student-Newman-Keuls post-hoc analysis, and defined as $p < 0.05$, with '*' vs. sham muscles from the same day. N=6-8 muscles/group.

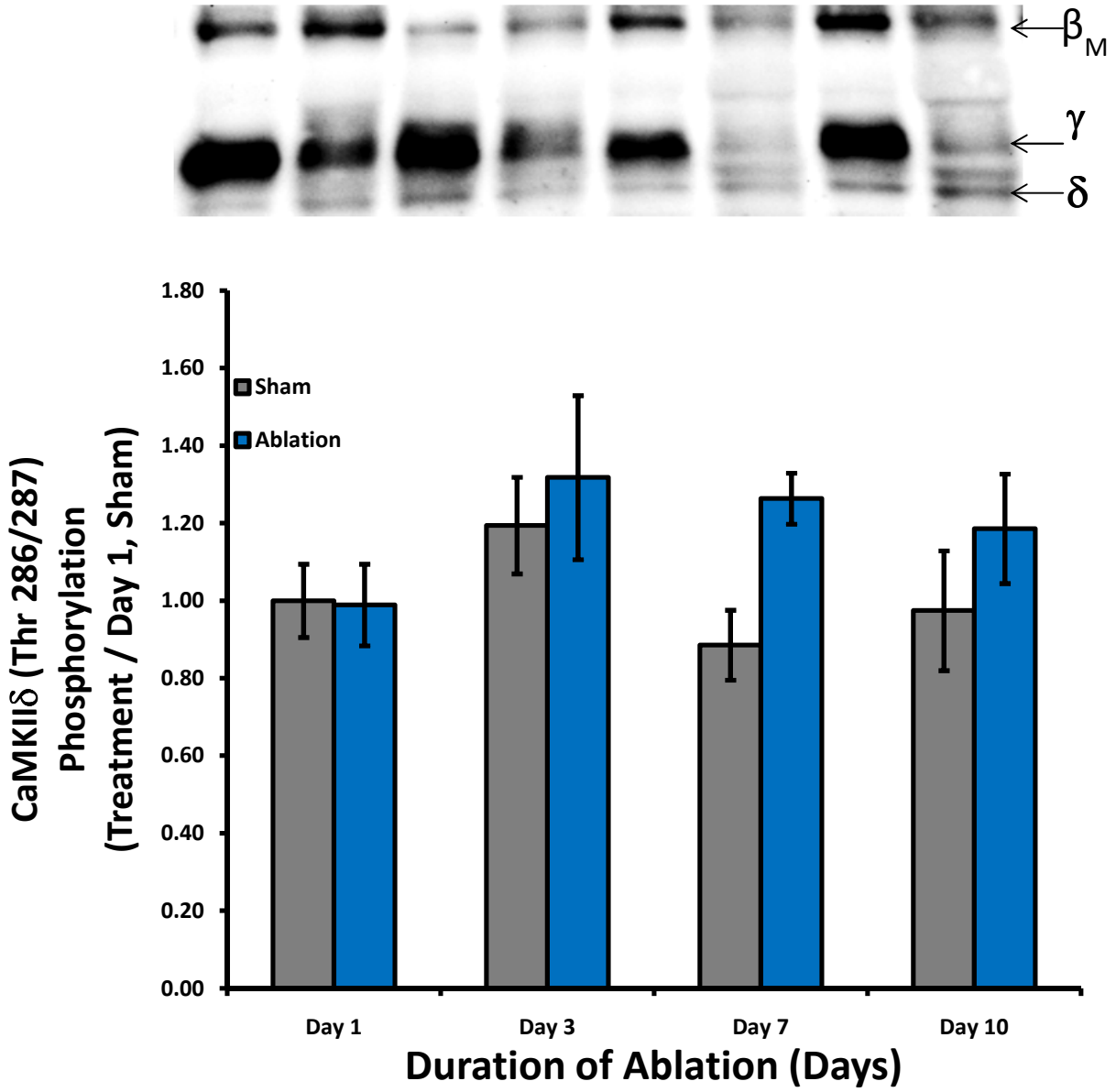
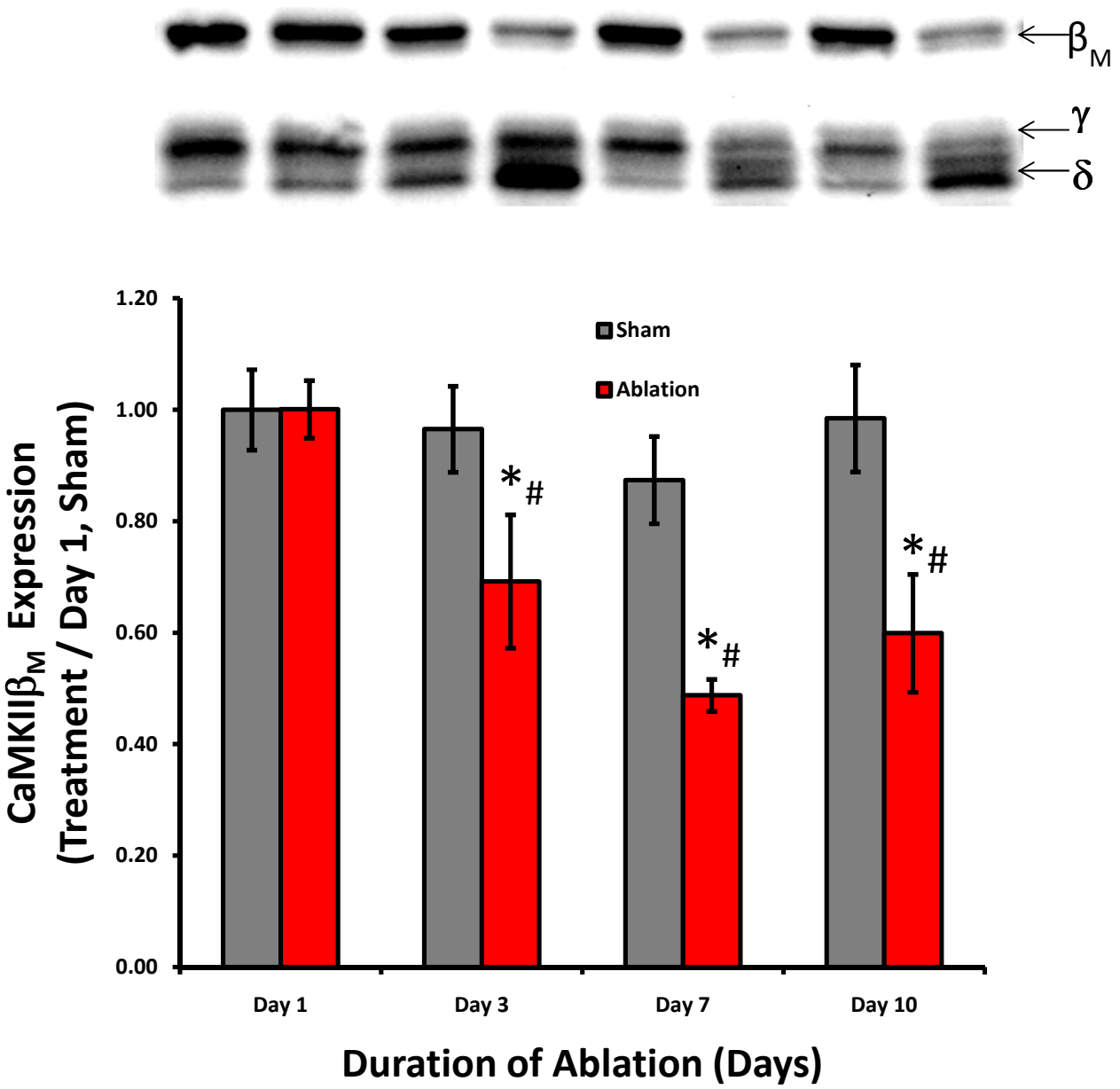
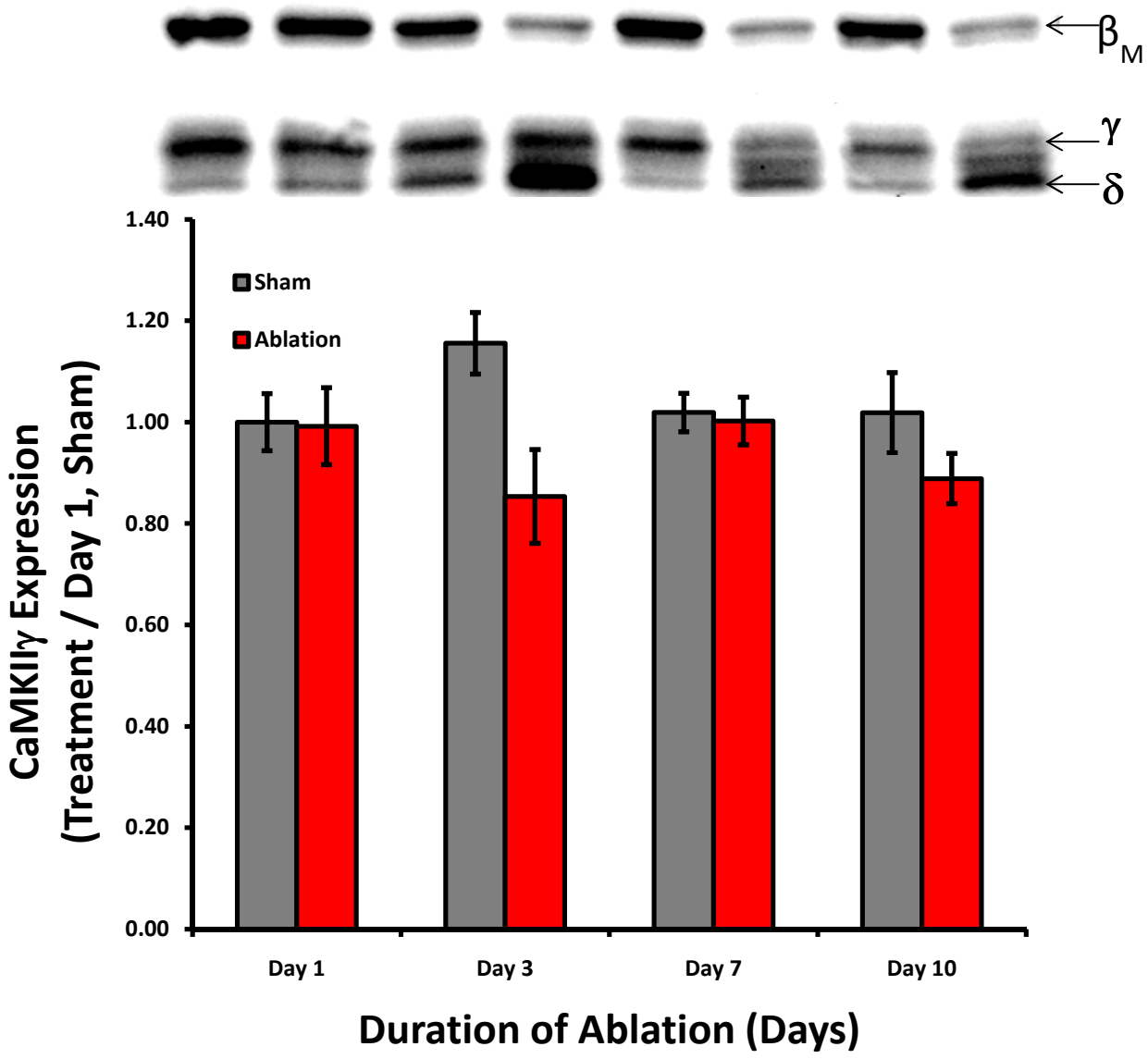


Figure 24: Effect of Synergist Ablation on CaMKIIδ Phosphorylation in Mouse Plantaris Muscles. Data are presented as the mean ± standard error of the mean. Statistical significance was assessed by Two-way ANOVA and Student-Newman-Keuls post-hoc analysis, and defined as $p < 0.05$. N=6-8 muscles/group.

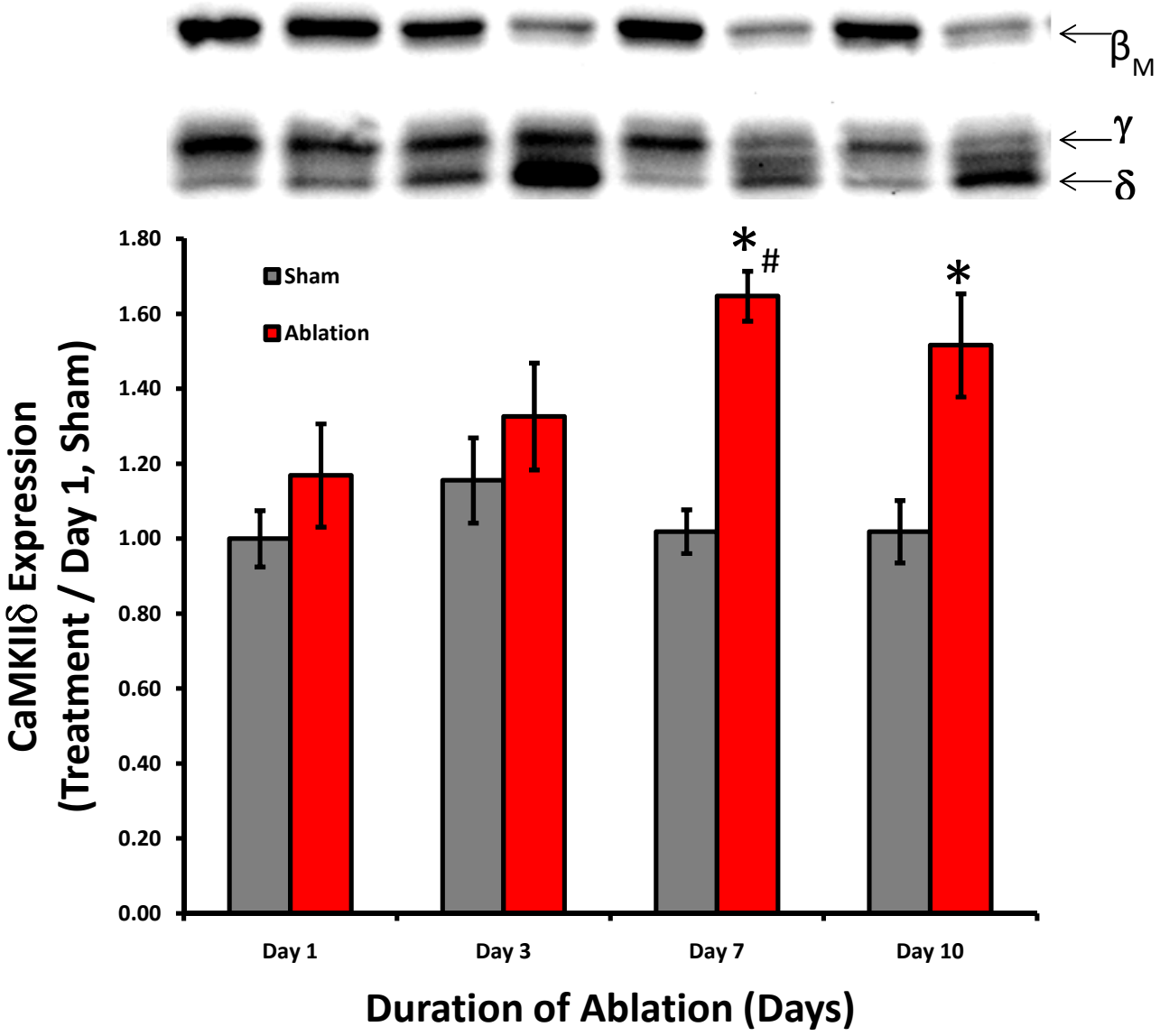
CaMKII Protein Expression. Figures 25 through 27 show representative blots and the quantification of band intensity for CaMKIIβ_M, CaMKIIγ and CaMKIIδ protein expression from the plantaris muscles of ablated and sham mouse legs. The overloaded plantaris muscles at 3, 7 and 10 days after the synergist ablation surgery had significantly lower CaMKIIβ_M protein expression compared to their sham plantaris muscles (Fig 25). Additionally, CaMKIIβ_M protein expression was significantly lower in overloaded plantaris muscles at days 3, 7 and 10 compared to CaMKIIβ_M protein expression in day 1 overloaded muscle (Fig 25). CaMKIIγ protein expression was not significantly altered by the synergist ablation surgery at any of the time points examined (Fig 26). Interestingly, CaMKIIδ protein expression was significantly higher (160% and 150%, respectively) in overloaded muscles 7 and 10 days after ablation compared to the sham muscles on the same days (Fig 27). Also, CaMKIIδ expression of day 7 overloaded plantaris muscles was significantly higher than the expression of day 1 overloaded muscles (Fig 27). CaMKIIβ_M, γ, and δ protein expression of the sham muscles was not significantly different at any time following synergist ablation (Fig 25,26,27).



<u>Figure 25</u>: Effect of Synergist Ablation on CaMKIIβ_M Protein Expression in Mouse Plantaris Muscles. Data are presented as the mean ± standard error of the mean. Statistical significance was assessed by Two-way ANOVA and Student-Newman-Keuls post-hoc analysis, and defined as $p < 0.05$, with '#' vs. Day 1 ablation and '*' vs. sham muscles on the same day. N=6-8 muscles/group.



<u>Figure 26</u>: Effect of Synergist Ablation on CaMKIIγ Protein Expression in Mouse Plantaris Muscles. Data are presented as the mean ± standard error of the mean. Statistical significance was assessed by Two-way ANOVA and Student-Newman-Keuls post-hoc analysis, and defined as $p < 0.05$. N=7-8 muscles/group.



<u>Figure 27</u>: Effect of Synergist Ablation on CaMKIIδ Protein Expression in Mouse Plantaris Muscles. Data are presented as the mean ± standard error of the mean. Statistical significance was assessed by Two-way ANOVA and Student-Newman-Keuls post-hoc analysis, and defined as $p < 0.05$, with '#' vs. Day 1 ablation and '*' vs. sham muscles on the same day. N=7-8 muscles/group.

Ratio of Phosphorylated CaMKII to Total CaMKII As previously mentioned in the "Denervation Surgery" section of this chapter, the ratio of phosphorylated CaMKII to CaMKII protein expression (phospho:total) was determined in order to gain a better perspective on overall activation of each CaMKII isoform.

Figures 28 through 30 show the ratios of CaMKII phosphorylation to CaMKII protein expression (phospho:total) for each isoform ($\beta_{M,}$ γ and δ) from the plantaris muscles of ablated and sham mouse legs. In Figure 28, the ratio of phospho:total CaMKIIβ_M was significantly higher in overloaded plantaris muscles 10 days after ablation compared to the 10 day sham muscles. The CaMKIIβ_M phospho:total ratio of overloaded plantaris muscles from day 10 was also significantly higher than overloaded plantaris muscles from day 1 (Fig 28). In contrast, the ratio of phospho:total CaMKIIγ were significantly decreased at 1, 3, 7 and 10 days after ablation compared to the sham muscles on the same day (Fig 29). Figure 30 shows no significant differences in the ratio of phospho:total CaMKIIδ.

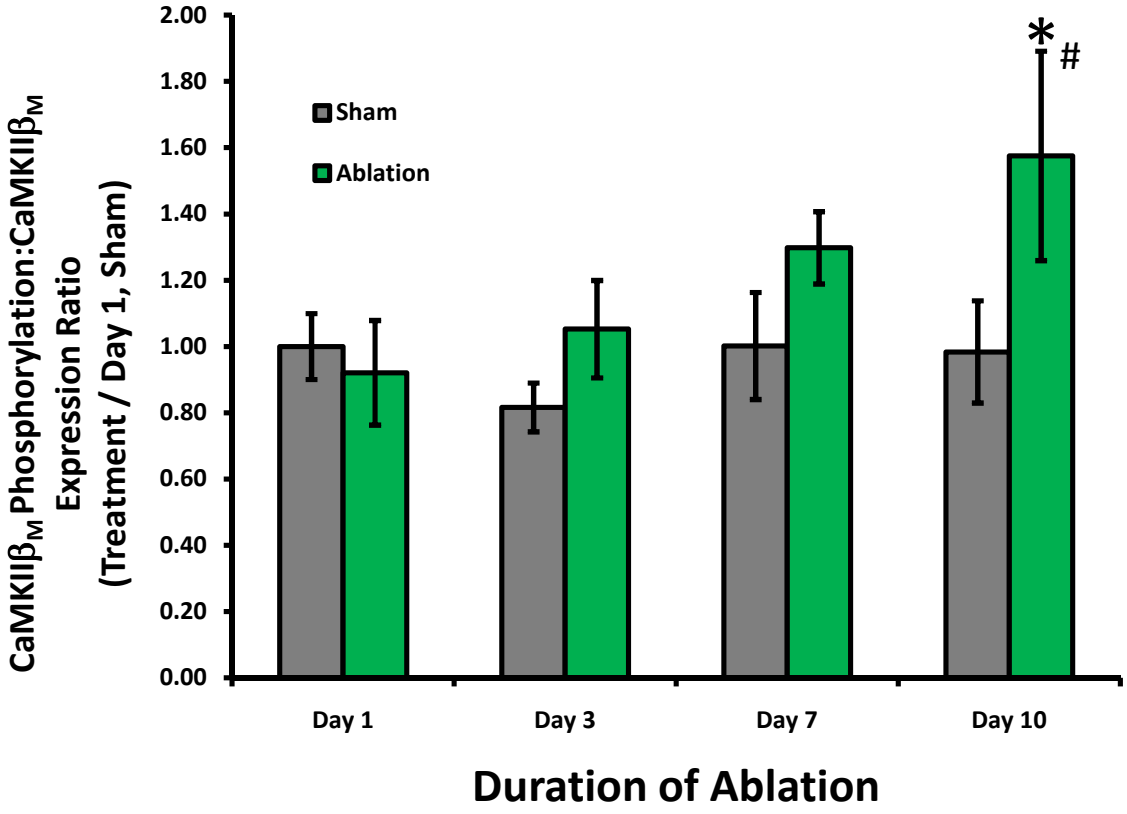
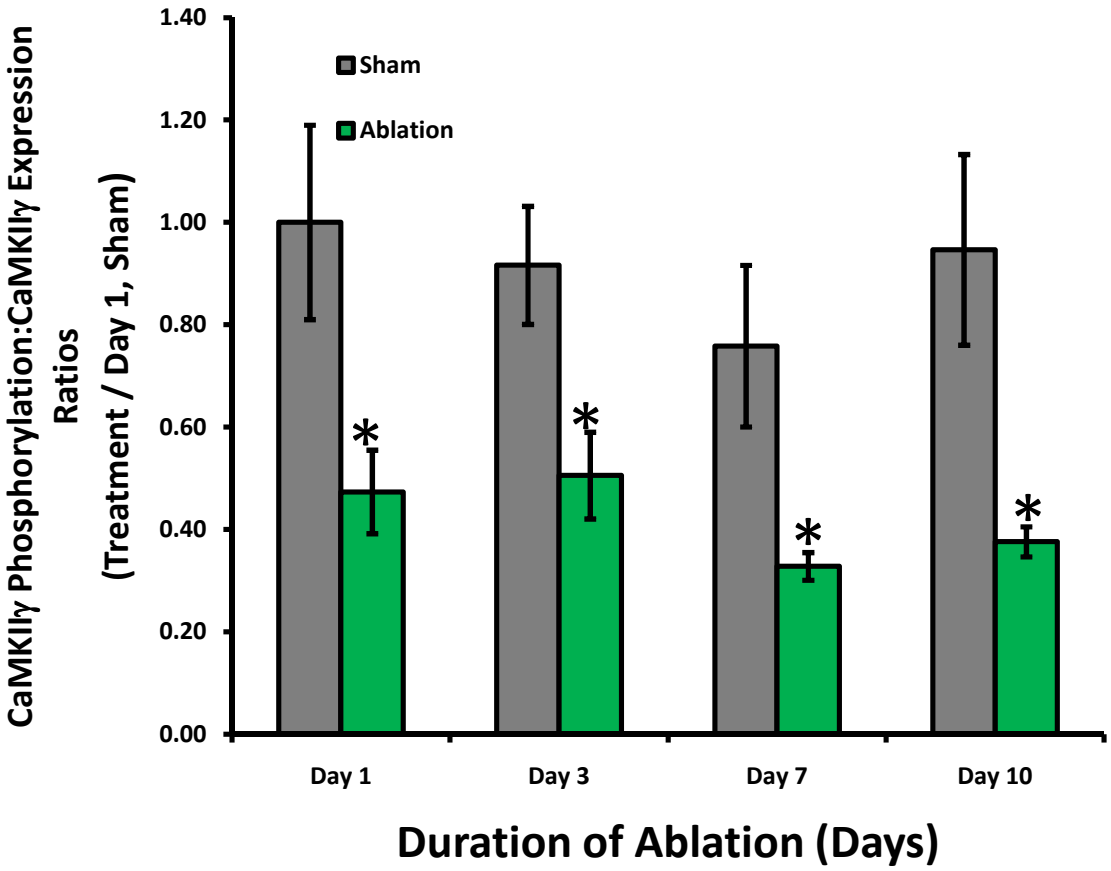
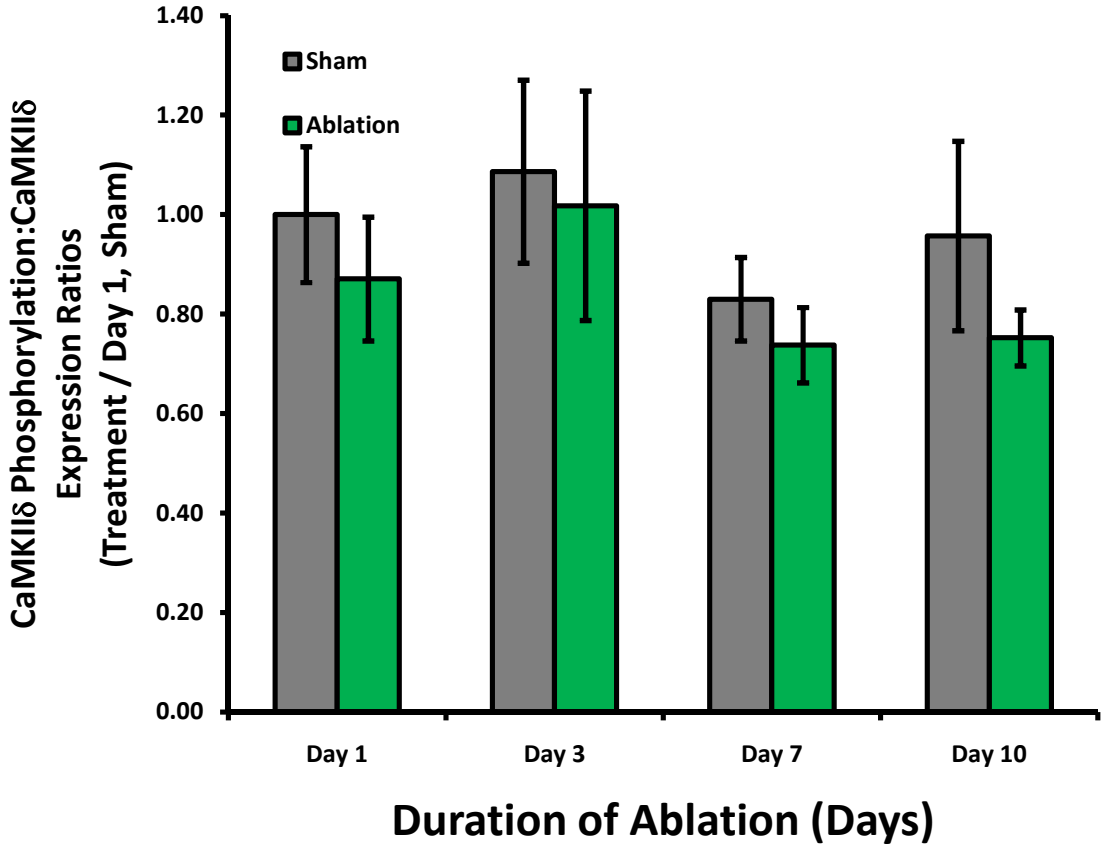


Figure 28: Effect of Synergist Ablation on the Ratios of CaMKIIβ$_M$ (Thr286/287) Phosphorylation to CaMKIIβ$_M$ Protein Expression in Mouse Plantaris Muscles. Data are presented as the mean ± standard error of the mean. Statistical significance was assessed by Two-way ANOVA and Student-Newman-Keuls post-hoc analysis, and defined as $p < 0.05$, with '#' vs. Day 1 Ablation and '*' vs. sham muscles from the same day. N=6-8 muscles/group.



<u>Figure 29</u>: Effect of Synergist Ablation on the Ratios of CaMKIIγ (Thr286/287) Phosphorylation to CaMKIIγ Protein Expression in Mouse Plantaris Muscles. Data are presented as the mean ± standard error of the mean. Statistical significance was assessed by Two-way ANOVA and Student-Newman-Keuls post-hoc analysis, and defined as $p < 0.05$, with '*' vs. sham muscles on the same day. N=6-8 muscles/group.



<u>Figure 30</u>: Effect of Synergist Ablation on the Ratios of CaMKIIδ (Thr286/287) Phosphorylation to CaMKIIδ Protein Expression in Mouse Plantaris Muscles. Data are presented as the mean ± standard error of the mean. Statistical significance was assessed by Two-way ANOVA and Student-Newman-Keuls post-hoc analysis, and defined as $p < 0.05$. N=6-8 muscles/group.

CHAPTER 5: DISCUSSION

Skeletal muscle is necessary for multiple vital functions in the human body, including movement, metabolism and respiration, and the ability of muscle to carry out these functions is dependent on the maintenance of skeletal muscle mass. Although the cellular and molecular mechanisms that regulate skeletal muscle mass are not completely known, preliminary studies have suggested a role for the Ca^{2+}-sensitive serine/threonine kinase, Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), in this process. In this study, we provide the first assessment of the time-dependent changes in CaMKIIβ_M, γ, and δ (Thr286/287) phosphorylation and protein expression in response to denervation-induced muscle atrophy and synergist ablation-induced muscle hypertrophy. Not only do these findings provide insight into the mechanisms regulating muscle mass, but they also provide a fundamental basis for potential future studies designed towards targeting CaMKII for the treatment of muscle wasting diseases.

In this study, polyclonal antibodies that are capable of identifying all known isoforms of CaMKII were utilized to examine CaMKII (Thr286/287) phosphorylation and CaMKII protein expression, with the CaMKIIβ_M, γ, and δ isoforms identified based on their expected molecular weights. Since we did not immunoblot with a monoclonal antibody specific for each isoform, it is possible that the bands defined as CaMKIIβ_M, CaMKIIγ and CaMKIIδ in this thesis may not be these three distinct CaMKII isoforms, but may instead represent splice variants of one or two of the CaMKII isoforms. Future experiments would need to be done to conclusively show that the bands detected by the polyclonal antibodies are indeed CaMKIIβ_M, CaMKIIγ and CaMKIIδ.

This is the first study to date that individually examined the phosphorylation and protein expression of the three major CaMKII isoforms found in skeletal muscle during atrophy and hypertrophy. Previous studies had either quantified the protein expression of the three isoforms

collectively (Witczak, unpublished observations, Fig 8), had focused on the expression of only one isoform (Chin, 2004; Fig 5), or had only examined CaMKII protein expression at one time point (Chin, 2004; Fluck et al., 2000; Stephens et al., 2010). Excitingly, our results showed that there are major differences in the phosphorylation and protein expression of CaMKIIβ_M, γ and δ during skeletal muscle atrophy and hypertrophy. For example, in our study, denervation induced a significant decrease in CaMKIIβ_M protein expression at 3, 7, 10 and 14 days (Fig 14), but an increase in CaMKIIγ and CaMKIIδ protein expression at 10 and 14 days (Figs 15 and 16). This divergence in response of the CaMKII isoforms underscores the importance of examining each isoform separately, and at different time points, since quantifying all of the isoforms together at a single time point could have resulted in a final conclusion of no change in CaMKII protein expression following loss of nerve activity to the muscle.

Previous work in rat skeletal muscle had shown that CaMKIIγ protein expression was increased ~2.5-fold following 20 days of denervation (Chin, 2004; Fig 5), suggesting that CaMKIIγ may play a role in the regulation of muscle atrophy. Consistent with that finding, in our study, we found that CaMKIIγ and CaMKIIδ protein expression increased following 10 and 14 days of denervation (Figs 15 and 16). Unfortunately, since these increases in CaMKIIγ and δ expression were not evident until 10 days after the denervation surgery, the data suggest that these changes in CaMKII expression are not initiating muscle atrophy following denervation. In Figures 15 and 16, the increase in CaMKIIγ and CaMKIIδ protein expression could be compensatory increases in order to offset the atrophic conditions (Chin, 2004; Stephens et al., 2009).

In contrast to the CaMKIIγ and δ isoforms, CaMKIIβ_M expression was significantly decreased following 3 days of denervation (Fig 14). Although previous muscle denervation

studies had attempted to examine CaMKIIβ_M expression, CaMKIIβ_M was not detected by the commercially-available antibodies at the time (Chin, 2004). Thus, this is the first report characterizing the time-dependent alterations in CaMKIIβ_M during denervation-induced muscle atrophy. Our observed decrease in CaMKIIβ_M protein expression was in contrast to a study that demonstrated an increase in CaMKIIβ mRNA and CaMKII protein levels in skeletal muscle from patients with cancer cachexia (Stephens, 2010). This difference in observed response of CaMKIIβ_M expression could be due to any of the following differences in model and/or methodology. First, cancer cachexia is a systemic syndrome that causes not only muscle wasting, but organ and other tissue wasting. While the exact mechanisms of cachexia are poorly understood, it is postulated that inflammation may be a major factor driving muscle atrophy (Stephens, 2010). Secondly, although CaMKII protein levels in cachexic patients increased, the specific CaMKII isoform that increased in expression was never specified. Finally, cachexia is a chronic syndrome in comparison to the 14 day atrophy time course performed in our research. In Figure 14, CaMKIIβ_M expression of 3 day denervated muscle was significantly lower compared to sham and day 1 denervated muscle and remained significantly lower through 10 days. However, after 14 days of denervation, CaMKIIβ_M expression increased to a level that was not significantly different than day 14 sham (Fig 14). Therefore, by the time CaMKII was quantified in the cachexic patients, expression could have significantly increased beyond control levels. Future studies that deal with muscle atrophy and CaMKIIβ expression could potentially benefit by increasing the duration of their time course.

Previous work in rat hippocampal neurons showed that elevations in intracellular Ca^{2+} levels or expression of a constitutively active form of CaMKII stimulated protein degradation, and these effects could be inhibited with the CaMK inhibitor, KN-93 (Djakovic et al., 2009).

Thus, these results suggested that phosphorylation and activation of CaMKII would be increased by denervation-induced muscle atrophy. Contrary to this work and our expected results, there were no significant changes in CaMKIIβ_M or CaMKIIδ (Thr286/287) phosphorylation (Figs 11,13), and CaMKIIγ phosphorylation was significantly decreased at 7, 10 and 14 days of denervation (Fig 12). There were some differences between our research and the research performed by Djakovic et al. that may explain this discrepancy. First, CaMKII$\beta_{M,}$ γ and δ are the major CaMKII isoforms found in skeletal muscle, while CaMKIIα is the major CaMKII isoform expression in the brain. Thus, perhaps there are isoform-specific functions in these two tissues that may account for the difference in finding. Second, we measured the expression and phosphorylation of each CaMKII isoform at multiple time points while Djakovic et al. measured the activity of CaMKIIα at one single time point (Fig 6,7). By observing changes in CaMKII at more than one time point, we were able to identify trends in expression and phosphorylation, thus allowing us to determine how each isoform differed from the other at multiple time points.

To date, only preliminary studies by Witczak CA (unpublished observations) have examined changes in CaMKII expression during skeletal muscle hypertrophy (Fig 8). These data showed that CaMKIIβ_M, γ and δ protein expression decreased 1 day after ablation and then increased to levels not significantly different from the sham muscles (Fig 8). Strikingly, in our results, the expression of each CaMKII isoform was distinctly different following synergist ablation surgery (Figs 25,26,27). CaMKIIγ protein expression did not change at any time point examined (Fig 26), CaMKIIβ_M expression significantly decreased after day 3 (Fig 25), and CaMKIIδ expression significantly increased after day 7 (Fig 27). The methods and materials utilized by Witczak CA are identical to ours with the exception of the strain of mouse (C57BL/6J). Therefore, the aforementioned differences between our CaMKII expression results

and the results from Witczak CA are confounding and may be attributed to the variation in mouse strain.

Although there is a lack of publications that suggest a role for CaMKII in skeletal muscle hypertrophy, there have been numerous studies performed looking at the role of CaMKII in cardiac muscle hypertrophy. Of particular interest to our research, studies have linked two splice variants of CaMKIIδ (CaMKIIδ_b and CaMKIIδ_c) to hypertrophy in cardiomyocytes (Anderson et al., 2011; Heineke and Molkentin, 2006; Huke et al., 2011; Maier, 2005; Pan et al., 2010). One study showed that transgenic mice overexpressing CaMKIIδ_c in the heart exhibited significant increases in myocardial hypertrophy and ventricular dysfunction (Zhang, 2005) while CaMKIIδ_b overexpression in cardiomyocytes increased the gene expression of atrial natriuretic factor, a well-established marker of ventricular hypertrophy (Ramirez et al, 1997). The increase in CaMKIIδ expression associated with cardiac hypertrophy is in agreement with our results showing that CaMKIIδ protein expression increased 7 and 10 days following synergist ablation-induced muscle hypertrophy (Fig 27). Furthermore, the increases in CaMKIIδ expression associated with both cardiac and skeletal muscle hypertrophy may indicate the muscle is attempting to increase protein synthesis following the introduction of a pathological or induced condition. This statement supports the hypothesis that the increases in CaMKIIδ expression following denervation-induced atrophy (Fig 16) are potentially due to a compensatory mechanism which is attempting to stimulate an increase in muscle mass and function (Chin, 2004; Stephens et al., 2009).

Our results are the first to show that muscle overload induces an immediate and sustained decrease in CaMKIIγ (Thr286/287) phosphorylation (Fig 23). Since neither CaMKIIβ_M nor CaMKIIδ (Thr286/287) phosphorylation was significantly altered in response to

the ablation surgery (Figs 22,24), these findings may indicate a change in CaMKIIγ intracellular localization during hypertrophy. This speculation is in agreement with previous literature that found that CaMKIIγ splice variants are localized to nuclei in brain tissue (Takeuchi et al., 1999). Thus, in our study, it is possible that the significant decrease in CaMKIIγ phosphorylation seen following ablation was due to nuclear translocation. In support of this speculation, our results have shown that CaMKIIγ in denervated skeletal muscle also displayed significant decreases in phosphorylation after 3 days (Fig 12). While there is no research to date that has conclusively defined a role for CaMKIIγ in the regulation of muscle mass, future studies may benefit by determining the effects of CaMKIIγ overexpression or CaMKIIγ gene knockout on muscle atrophy and hypertrophy.

In summary, the goal of this study was to determine whether one or more of the CaMKII isoforms present in skeletal muscle is altered in response to atrophic or hypertrophic stimuli and the possible time dependency of those changes. Collectively, our results showed that the phosphorylation and the protein expression of CaMKIIβ_M, γ or δ are differentially regulated following denervation-induced muscle atrophy and synergist ablation-induced hypertrophy. These findings underscore the necessity of examining each CaMKII isoform separately in order to determine its possible role in regulating muscle mass.

References

Akimoto T, Ribar TJ, Williams RS, Yan Z. Skeletal muscle adaptation in response to voluntary running in Ca2+/calmodulin-dependent protein kinase IV-deficient mice. Am J Physiol Cell Physiol. 287(5): C1311-9, 2004.

Al-Shanti N and Stewart CE. Ca^{2+}/Calmodulin-dependent transcriptional pathways: potential Mediators of skeletal muscle growth and development. Biol. Rev. 84: 637-652, 2009.

Anderson ME, Braun AP, Schulman H and Premack BA. Multifunctional Ca^{2+}/calmodulin-Dependent protein kinase mediates Ca^{2+}-induced enhancement of the L-type Ca^{2+} current in rabbit ventricular myocytes. Circ. Res. 75: 854-915, 1994.

Anderson ME, Brown JH, Bers DM. CaMKII in myocardial hypertrophy and heart failure. J Mol Cell Cardiol. 51(4):468-73, 2011.

Baechle TR and Earle RW. Essentials of Strength and Conditioning. 3:4-8; 183-191, 2008

Baracos VE, DeVivo C, Hoyle DH. Activation of the ATP-ubiquitin-proteasome pathway in skeletal muscle of cachectic rats bearing a hepatoma. Am J Physiol 268 (5 Pt. 1): E996–E1006, 1995

Barber MD, Ross JA, Fearon KC. Cancer cachexia. Surg Oncol 8:133–41, 1999.

Bodine SC, Latres E, Baumhueter S, Lai VK, Nunez L, Clarke BA, Poueymirou WT, Panaro FJ, Na E, Dharmarajan K, Pan ZQ, Valenzuela DM, DeChiara TM, Stitt TN, Yancopoulos GD, Glass DJ. Identification of ubiquitin ligases required for skeletal muscle atrophy. Science 294: 1704–1708, 2001a.

Bodine SC, Stitt TN, Gonzalez M, Kline WO, Stover GL, Bauerlein R, Zlotchenko E, Scrimgeour A, Lawrence JC, Glass DJ, Yancopoulos GD. Akt/mTOR pathway is a crucial regulator of skeletal muscle hypertrophy and can prevent muscle atrophy in vivo. Nat Cell Biol 3: 1014–1019, 2001b.

Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 72:248-54, 1976.

Braun AP and Schulman H. The multifunctional calcium/calmodulin-dependent protein kinase: from form to function. Annual Review of Physiology. 57: 417–445, 1995.

Castro, MJ., Apple Jr., DF., Staron, RS., Campos GER. and Dudley, GA. Influence of Complete Spinal Cord Injury on Skeletal Muscle within 6 months of Injury. J. Appl. Physiol. 86: 350-358, 1999.

Chin ER. The Role of Calcium and Calcium/Calmodulin-Dependent Kinases in Skeletal Muscle Plasticity and Mitochondrial Biogenesis. Proceedings of the Nutrition Society. 63(2): 279-287, 2004.

Ciechanover A. The ubiquitin-proteasome proteolytic pathway. Cell 79:13–21, 1994.

DeKoninck P and Schulman H. Sensitivity of CaM kinase II to the frequency of Ca^{2+} Oscillations. Science. 279: 227-257, 1998.

Djakovic SN, Schwarz LA, Barylko B, DeMartino GN and Patrick GN. Regulation of the proteasome by neuronal activity and calcium/calmodulin protein kinase II. J Biol. Chem. 284(39): 26655-26665, 2009.

Evans WJ. Skeletal Muscle Loss: Cachexia, Sarcopenia, and Inactivity. Am. J. Clin. Nutr. 91(4): 1123S-1127S, 2010.

Fluck M, Waxman MN, Hamilton MT and Booth FW. Skeletal muscle Ca^{2+} -independent kinase Activity increases during either hypertrophy or running. J Appl. Phsiol. 88: 352-360, 2000.

Giangregorio, L and McCartney, N. Bone Loss and Muscle Atrophy in Spinal Cord Injury: Epidemiology, Fracture Prediction, and Rehabilitation Strategies. J. Spinal Cord Med. 29(5): 489-500, 2006.

Gomes MD, Lecker SH, Jagoe RT, Navon A, Goldberg AL. Atrogin-1, a muscle-specific F-box protein highly expressed during muscle atrophy. Proc Natl Acad Sci *USA* 98: 14440–14445, 2001.

Häkkinen K, Alen M, Kraemer WJ, Gorostiaga E, Izquierdo M, Rusko H, Mikkola J, Häkkinen A, Valkeinen H, Kaarakainen E, Romu S, Erola V, Ahtiainen J, Paavolainen L. Neuromuscular adaptations during concurrent strength and endurance training versus strength training. Eur J Appl Physiol 89(1):42-52, 2002.

Hartl F. Molecular chaperones in cellular protein folding. Nature 381:571–579, 1996

Heineke J, Molkentin JD. Regulation of cardiac hypertrophy by intracellular signaling pathways. Nat Rev Mol Cell Biol. (8):589-600, 2006.

Hudmon A, Schulman H. Structure-function of the multifunctional Ca2+/calmodulin-dependent protein kinase II. Biochem J. 364(Pt. 3): 593-611, 2002.

Huke S, Desantiago J, Kaetzel MA, Mishra S, Brown JH, Dedman JR, Bers DM. SR-targeted CaMKII inhibition improves SR Ca^{2+} handling, but accelerates cardiac remodeling in mice overexpressing CaMKIIδC. J Mol Cell Cardiol. 50(1):230-8, 2011.

Ishida A, Okuno S, Kitani T, Kameshita I, Fujisawa H. Regulation of multifunctional Ca2+/calmodulin-dependent protein kinases by Ca2+/calmodulin-dependent protein kinase phosphatase. Biochem Biophys Res Commun. 253(1):159-63, 1998.

Ishikawa Y, Tokumitsu H, Inuzuka H, Murata-Hori M, Hosoya H, Kobayashi R. Identification and characterization of novel components of a Ca2+/calmodulin-dependent protein kinase cascade in HeLa cells. FEBS Lett. 550(1-3): 57-63, 2003.

Jones SW, Hill RJ, Kransey, O'Conner B, Pierce N and Greenhaff PL. Disuse Atrophy and Exercise Rehabilitation Affects the Expression of Genes Associated with the Regulation of Skeletal Muscle Mass. FASEB J. April 14, 2004

Juretic A, Spagnoli GC, Horig H. Glutamine requirements in the generation of lymphokine-activated T cells. Clin Nutr 13:42–49, 1994.

Kamata A, Sakagami H, Tokumitsu H, Owada Y, Fukunaga K, Kondo H. Spatiotemporal expression of four isoforms of Ca2+/calmodulin-dependent protein kinase I in brain and its possible roles in hippocampal dendritic growth. Neurosci Res. 57(1): 86-97, 2007.

Kamei Y, Miura S, Suzuki M, Kai Y, Mizukami J, Taniguchi T, Mochida K, Hata T, Matsuda J, Aburatani H, Nishino I, Ezaki O. Skeletal muscle FOXO1 (FKHR) transgenic mice have less skeletal muscle mass, down-regulated type I (slow twitch/red muscle) fiber genes, and impaired glycemic control. J Biol Chem 279: 41114–41123, 2004.

Lecker SH, Jagoe RT, Gilbert A, Gomes M, Baracos V, Bailey J, Price SR, Mitch WE, Goldberg AL. Multiple Types of Skeletal Muscle Atrophy Involve a Common Program of Changes in Gene Expression. FASEB J. 18: 39-51, 2004.

Lee SW, Dai G, Hu Z, Wang X, Du J, Mitch WE. Regulation of muscle protein degradation: coordinated control of apoptotic and ubiquitin-proteasome systems by phosphatidylinositol 3 kinase. J Am Soc Nephrol 15: 1537–1545, 2004.

Lightfoot, A., McArdle, A. and Griffiths, RD. Muscle in Defense. Critical Care Medicine. 37(10): S384-S390, 2009.

Maier LS. CaMKIIdelta overexpression in hypertrophy and heart failure: cellular consequences for excitation-contraction coupling. Braz J Med Biol Res. 38(9):1293-302, 2005.

McArdle F, Spiers S, Aldemir H. Preconditioning of skeletal muscle against contraction-induced damage: The role of adaptations to oxidants in mice. J Physiol 561:233–244, 2004

McCall GE, Allen DL, Haddad F, Baldwin KM. Transcriptional regulation of IGF-I expression In skeletal muscle. Am J Physiol Cell Physiol 285: C831–C839, 2003.

Mishra S, Gray CB, Miyamoto S, Bers DM, Brown JH. Location matters: clarifying the concept of nuclear and cytosolic CaMKII subtypes. Circ Res. 109(12):1354-62, 2011.

Muscaritoli M., Bossola M., Aversa Z., Bellantone R. Fanell, FR. Prevention and Treatment of Cancer Cachexia: New Insights into an Old Problem. European Journal of Cancer. 42: 31-41, 2006.

Musaro A, McCullagh K, Paul A, Houghton L, Dobrowolny G, Molinaro M, Barton ER, Sweeney HL, Rosenthal N. Localized IGF1 transgene expression sustains hypertrophy and regeneration in senescent skeletal muscle. Nat Genet 27: 195–200, 2001.

Ohanna M, Sobering AK, Lapointe T, Lorenzo L, Praud C, Petroulakis E, Sonenberg N, Kelly PA, Sotiropoulos A, Pende M. Atrophy of S6K1 (–/–) skeletal muscle cells reveals distinct mTOR effectors for cell cycle and size control. Nat Cell Biol 7: 286–294, 2005.

Pallafacchina G, Calabria E, Serrano AL, Kalhovde JM, Schiaffino S. A protein kinase B-dependent and rapamycin-sensitive pathway controls skeletal muscle growth but not fiber type specification. Proc Natl Acad Sci USA 99: 9213–9218, 2002.

Pan ZW, Zhang Y, Mei DH, Zhang R, Wang JH, Zhang XY, Xu CQ, Lu YJ, Yang BF. Scutellarin exerts its anti-hypertrophic effects via suppressing the Ca2+-mediated calcineurin and CaMKII signaling pathways. Naunyn Schmiedebergs Arch Pharmacol. (2):137-45, 2010.

Pelosi M and Donella-Deana A. Localization, purification, and characterization of the rabbit sarcoplasmic reticulum associated calmodulin-dependent protein kinase. Biochemistry. 65: 259-327, 2000.

Ramirez MT, Zhao XL, Schulman H, Brown JH. The nuclear deltaB isoform of Ca/calmodulin-dependent protein kinase II regulates atrial natriuretic factor gene expression in ventricular myocytes. J Biol Chem. 272(49):31203–8, 1997.

Rose AJ, Alsted TJ, Kobberø JB, Richter EA. Regulation and function of Ca2+-calmodulin-dependent protein kinase II of fast-twitch rat skeletal muscle. J Physiol. 580(Pt.3): 993-1005, 2007a.

Rose AJ, Frøsig C, Kiens B, Wojtaszewski JF, Richter EA. Effect of endurance exercise training on Ca2+ calmodulin-dependent protein kinase II expression and signalling in skeletal muscle of humans. J Physiol. 583(Pt. 2):785-95, 2007b.

Roy, RR and Edgerton, VR. Response of Mouse Plantaris Muscle to Functional Overload: Comparison with Rat and Cat. Comp. Biochem. Physiol. 111A(4): 569-575, 1995.

Sandri M. Signaling in Muscle Atrophy and Hypertrophy. Physiology 23: 160-170, 2008

Sandri M, Sandri C, Gilbert A, Skurk C, Calabria E, Picard A, Walsh K, Schiaffino S, Lecker SH, Goldberg AL. Foxo transcription factors induce the atrophy-related ubiquitin ligase atrogin-1 and cause skeletal muscle atrophy. Cell 117: 399–412, 2004.

Saucerman JJ and Bers DM. Calmodulin mediates differential sensitivity of CaMKII and calcineurin to local Ca^{2+} in cardiac myocytes. Biophys J. 95(10): 4597-612, 2008.

Schiaffino S and Reggiani C. Molecular diversity of myofibrillar proteins: gene regulation and functional significance. Physiol. Rev. 76: 371-794, 1996.

Shah,OJ, Anthony,JC, Kimball,SR, Jefferson,LS: 4E-BP1 and S6K1: translational integration sites for nutritional and hormonal information in muscle. Am.J Physiol Endocrinol.Metab 279:E715-E729, 2000.

Shavlakadze T, White JD, Davies M, Hoh JFY and Grounds MD. Insulin-Like Growth Factor I Slows the Rate of Denervation Induced Muscle Atrophy. Neuromuscular Disorders. 15: 139-146, 2005.

Singh P, Salih M, Leddy JJ, Tuana BS. The muscle-specific calmodulin-dependent protein kinase assembles with the glycolytic enzyme complex at the sarcoplasmic reticulum and modulates the activity of glyceraldehyde-3-phosphate dehydrogenase in a Ca2+/calmodulin-dependent manner. J Biol Chem. 279(34):35176-82, 2004.

Soderling TR, Chang B and Brickey D. Cellular signaling through multifunctional Ca^{2+}/calmodulin-dependent protein kinase II. Journal of Biological Chemistry. 276: 3719–3722, 2001.

Southgate RJ, Neill B, Prelovsek O, El-Osta A, Kamei Y, Miura S, Ezaki O, McLoughlin TJ, Zhang W, Unterman TG, Febbraio MA. FOXO1 regulates the expression of 4E-BP1 and inhibits mTOR signaling in mammalian skeletal muscle. J Biol Chem 282: 21176–21186, 2007.

Spittler A, Winkler S, Gotzinger P. Ingluence of glutamine on the phenotype and function of human monocytes. Blood 86:1564–1569, 1995.

Stefan MI, Edelstein SJ, Le Novère N. An allosteric model of calmodulin explains differential Activation of PP2B and CaMKII. Proc Natl Acad Sci U S A. 105(31): 10768-73, 2008.

Stephens NA, Gallagher IJ, Rooyackers O, Skipworth RJ, Tan BH, Marstrand T, Ross JA, Guttridge DC, Lundell L, Fearon KC, Timmons JA. Using transcriptomics to identify and validate novel biomarkers of human skeletal muscle cancer cachexia. Genome Med. Jan 15;2(1):1, 2010.

Takata,M, Ogawa,W, Kitamura,T, Hino,Y, Kuroda,S, Kotani,K, Klip,A, Gingras,AC, Sonenberg,N, Kasuga,M: Requirement for Akt (protein kinase B) in insulin-induced activation of glycogen synthase and phosphorylation of 4E-BP1 (PHAS-1). J Biol.Chem. 274:20611-20618, 1999.

Takeuchi Y, Yamamoto H, Matsumoto K, Kimura T, Katsuragi S, Miyakawa T, Miyamoto E. Nuclear localization of the delta subunit of Ca2+/calmodulin-dependent protein kinase II in rat cerebellar granule cells. J Neurochem. 72(2):815-25, 1999.

Terada,Y, Tomita,K, Homma,MK, Nonoguchi,H, Yang,T, Yamada,T, Yuasa,Y, Krebs,EG, Sasaki,S, Marumo,F: Sequential activation of Raf-1 kinase, mitogen-activated protein (MAP) kinase kinase, MAP kinase, and S6 kinase by hyperosmolality in renal cells. J.Biol.Chem. 269:31296-31301, 1994.

Thomson, DM and Gordon, SE. Diminished overload-induced hypertrophy in aged fast-twitch skeletal muscle is associated with AMPK hyperphosphorylation. J Appl Physiol. 98(2): 557-64, 2005.

Thomson, DM and Gordon, SE. Impaired Overload-Induced Muscle Growth is Associated with Diminished Translational Signaling in Aged Rat Fast-Twitch Skeletal Muscle. Journal of Physiology. 574(1): 291-305, 2006.

Tokumitsu H, Hatano N, Inuzuka H, Yokokura S, Nozaki N, Kobayashi R. Mechanism of the generation of autonomous activity of Ca2+/calmodulin-dependent protein kinase IV. J Biol Chem. 279(39): 40296-302, 2004

Witczak CA, Fujii N, Hirshman MF, Goodyear LJ. Ca^{2+}/calmodulin-dependent protein kinase kinase-alpha regulates skeletal muscle glucose uptake independent of AMP-activated protein kinase and Akt activation. Diabetes. 56(5): 1403-9, 2007.

Witczak CA, Jessen N, Warro DM, Toyoda T, Fujii N, Anderson ME, Hirshman MF, Goodyear LJ. CaMKII Regulates Contraction- but not Insulin-Induced Glucose Uptake in Mouse Skeletal Muscle. Am. J. Physiol. Endocrinol Metab. 298: E1150-E1160, 2010.

Yan, Z., Okutsu, M., Akhtar, NY. and Lira, VA. Regulation of Exercise-Induced Fiber Type Transformation, Mitochondrial Biogenesis, and Angiogenesis in Skeletal Muscle. J. Appl. Physiol. October 28, 2010.

Zhang T, Maier LS, Dalton ND. The δC isoform of CaMKII is activated in cardiac hypertrophy and induces dilated cardiomyopathy and heart failure. Circulation Research. 92: 912-919, 2003.

APPENDIX A

East Carolina University

Animal Care and Use Commitee
212 Ed Warren Life Sciences Building
East Carolina University
Greenville, NC 27834

252-744-2436 office
252-744-2355 fax

November 1, 2010

Jeffrey Brault, Ph.D.
Department of EXSS
Ward Sports Medicine Bldg.
East Carolina University

Dear Dr. Brault:

Your Animal Use Protocol entitled, "Impaired Cellular Energetics and Atrophy of Skeletal Muscle" (AUP #P063) was reviewed by this institution's Animal Care and Use Committee on 11/1/10. The following action was taken by the Committee:

"Approved as submitted"

Please contact Dale Aycock at 744-2997 prior to hazard use

A copy is enclosed for your laboratory files. Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol. Modifications of these procedures cannot be performed without prior approval of the ACUC. The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies.

Sincerely yours,

Robert G. Carroll, Ph.D.
Chairman, Animal Care and Use Committee

RGC/jd

enclosure