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Increased Expression of δCaMKII Isoforms in Skeletal Muscle Regeneration: Implications in Dystrophic Muscle Disease

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Abstract The expression of δ isoforms of calcium-calmodulin/dependent protein kinase II (CaMKII) has been reported in mammalian skeletal muscle; however, their functions in this tissue are largely unknown. This study was conducted to determine if δCaMKII expression was altered during regeneration of skeletal muscle fibers in two distinct models. In the first model, necrosis and regeneration were induced in quadriceps of normal mice by intramuscular administration of 50% glycerol. Immunostaining and confocal microscopy revealed that δCaMKII expression was clearly enhanced in fibers showing centralized nuclei. The second model was the *mdx* mouse, which undergoes enhanced muscle necrosis and regeneration due to a mutation in the dystrophin gene. sern blot analysis of hind leg extracts from 4 to 6 week old *mdx* mice revealed that δCaMKII content was decreased when compared to age-matched control mice. This loss in δ kinase content was seen in myofibrillar and membrane fractions and was in contrast to unchanged δCaMKII levels in cardiac and brain extracts from dystrophic mice. Confocal microscopy of *mdx* quadriceps and tibialis muscle showed that δCaMKII expression was uniformly decreased in most fibers from dystrophic mice; however, enhanced kinase expression was observed in regenerating muscle fibers. These data support a fundamental role for δCaMKII in the regeneration process of muscle fibers in normal and *mdx* skeletal muscle and may have important implications in the reparative process following muscle death. J. Cell. Biochem. 97: 621–632, 2006.

Key words: skeletal; muscle; CaMKII; expression; mdx; regeneration

Calcium-calmodulin/dependent protein kinase II (CaMKII) is a ubiquitous serine/threonine protein kinase that appears to have a myriad of functions by virtue of the large number of purported substrates identified [Braun and Schulman, 1995]. Four distinct genes encode for mammalian CaMKII resulting in α, β, δ, and γ isoforms with multiple splice variants and exhibiting distinct tissue distribution [Braun and Schulman, 1995; Singer et al., 1996]. The expression of α and β CaMKII isoforms is primarily restricted to neuronal tissue (with notable exceptions), while δ and γ isoforms have a more widespread distribution [Braun and Schulman, 1995; Singer et al., 1996]. Despite significant diversity of CaMKII isoform expression and distribution the regulation of its activity or substrate specificity appears similar. In the presence of free intracellular calcium and calmodulin, CaMKII undergoes an autophosphorylation reaction that results in activation of the kinase domain [Braun and Schulman, 1995; Singer et al., 1996, Abraham et al., 1996], that phosphorylates specific cellular targets. Thus, the functions of various CaMKII isoforms are presumably regulated by tissue specific expression as well as discrete localization within the cell [Bayer et al., 1998].

A variety of molecular and biochemical studies have demonstrated that mammalian skeletal muscle expresses CaMKII. Early studies by Chu et al. [1990] described a CaM kinase activity that was associated with the junctional sarcoplasmic reticulum of rabbit skeletal muscle that phosphorylated the ryanodine receptor in vitro. A series of investigations by

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Madhavan and Jarrett [1994, 1999] identified a CaMKII activity associated with the dystrophin-glycoprotein complex (DGC) of skeletal muscle. These authors reported that in vitro phosphorylation of α-syntrophin by purified brain CaMKII decreased the binding of α-syntrophin to dystrophin (1999). Molecular cloning experiments have identified the expression of unique δ_4, β_M, and γ_B CaMKII isoforms in mammalian skeletal muscle [Schworer et al., 1993; Bayer et al., 1998]. In the case of the β_M isoform Bayer et al. [1998] characterized a novel anchoring protein (αKAP) that appeared to target the kinase to the sarcoplasmic reticulum of skeletal fibers. Work by Karczewski's group has described the expression of additional variants of δCaMKII in striated muscle that are associated with the embryonic developmental program in cardiac and skeletal muscle [Hagemann et al., 1999; Hoch et al., 2000]. Additionally, human skeletal muscle appears to express analogous CaMKII isoforms to the β_M and δ and/or γ isoforms that have been identified in other mammalian muscle [Rose and Hargreaves, 2003]. The above studies would suggest that skeletal muscle expresses distinct isoforms of CaMKII with unique functions; however these functions are largely unknown.

The current studies were undertaken to determine if the "embryonic" δCaMKII isoforms described previously [Hagemann et al., 1999; Hoch et al., 2000] were differentially regulated in two distinct models of skeletal muscle regeneration. The first model of regeneration utilized in this study was produced by intramuscular glycerol injections in normal mice causing acute necrosis and subsequent myofiber regeneration and muscle repair [Kawai et al., 1990]. The second is the *mdx* mouse, which undergoes a well-defined process of muscle necrosis and regeneration due to a point mutation in the dystrophin gene. Our study demonstrates that the expression of δCaMKII is markedly enhanced in replicating myoblasts and regenerating myofibers after glycerol-induced necrosis. We also provide evidence that a similar increase in δCaMKII expression is observed in regenerating myofibers from the *mdx* mouse. These results suggest a critical role for δCaMKII isoforms in the regenerative processes of skeletal muscle and may have important implications in muscle repair due to disease or injury.

MATERIALS AND METHODS

Materials

The CK2-Delta polyclonal antibody was a kind gift from Dr. H. Singer (Albany Medical College, Albany), while monoclonal antibodies for desmin were from Sigma Chemical Co. (St. Louis). Anti-δCaMKII antibody (A-17, goat polyclonal) was obtained from Santa Cruz Biotechnology (Santa Cruz). Anti-IgG antibodies coupled to HRP and ECL reagent for immunoblotting were from Amersham Pharmacia (Piscataway), while anti-IgGs coupled to AlexaFluor 488 or 546 were obtained from Molecular Probes (Eugene). Unless indicated otherwise all other chemicals and supplies were from Sigma Chemical Co.

Skeletal Muscle Tissue Isolation

Male *mdx* mice (C57BL/10SnJDMD^{mdx}) and their genetic control (C57BL/10SnJ), originally obtained from Jackson Laboratories (Bar Harbor, ME), were bred in the Campbell University Animal Care Facility. Four to six-week old animals were primarily used in this study since evidence of skeletal muscle necrosis and regeneration are most obvious during this period [Cooper, 2001]. Animal use in these studies was conducted under the authorization of the Campbell University IACUC. The animals were euthanized by CO_2 anesthesia and cervical dislocation before tissues were dissected out for experimental use.

Glycerol-Induced Necrosis

Skeletal fibers were induced to undergo necrosis and regeneration by the method described by Kawai et al. [1990]. The quadriceps muscle of 4-week-old C57Bl10SnJ mice were injected with 50 μL of 50% glycerol (saline, v/v). The contralateral tissue received an equivalent volume of saline and the animals were allowed to recover for 7 days before the muscles were removed and prepared for hematoxylin/eosin or immunostaining. The data in the figures are representative of three separate animals in each treatment group.

Confocal Microscopy

The hind legs of *mdx* and control mice were cryoprotected overnight at 4°C in phosphate-buffered saline (PBS) containing 30% sucrose prior to sectioning. Quadriceps and anterior tibialis muscle from the hind limbs were

mounted in Neg50 (Richard-Allen Scientific, MI) and quick-frozen at −50°C in a MicrOM cryostat (Walldorf, Germany) before 10 μm sections were cut and thaw-mounted onto glass slides. Sections were fixed with ice-cold methanol for 5 min before standard hematoxylin/eosin staining or immunostaining were performed. Cryosections for immunostaining were blocked with 1% bovine serum albumin in PBS prior to incubation with antibodies. Sections were exposed to primary antibodies (diluted in 1% BSA-PBS) against δCaMKII (rabbit polyclonal CK2-Delta at 1:80 or goat polyclonal A-17 at 1:25 dilution) or desmin (monoclonal, 1:200 dilution) at 37°C for 1 h. The primary antibody stain was detected by appropriate anti-IgG secondary antibodies coupled to AlexaFluor 488 or 546 (1:300 dilution). Specificity of the primary antibodies was determined by similar staining patterns obtained from antibodies directed against distinct epitopes of the same protein for example CK2-delta versus A17. Additional controls for immunostaining were performed by leaving out the primary or secondary antibody to account for any non-specific binding. The ability of CK2-Delta antibody to selectively stain for δCaMKII in situ has also been demonstrated by Van Riper et al. [2000]. Where appropriate the nuclei in the tissue sections were stained by 10-min incubation with 1 μg/ml propidium iodide prior to mounting the sections with Fluoromount-G (Southern Biotech, Birmingham). Immunostaining of cell structures was visualized by an excitation line from 488 nm argon or 546 nm helium-neon lasers and acquired by an iX70 microscope and Fluoview 300 confocal system (Olympus, NY). During image acquisition all settings such as gain, photomultiplier voltage, laser power, etc. were kept constant in order to objectively compare staining intensities between dystrophic (or treated) and normal tissues. Hematoxylin/eosin stain of sections was imaged by a CCD camera on an iX70 microscope (Olympus) and acquired using Microsuite software (Olympus). The original 12-bit multi-TIF images were converted to 8-bit TIF images to be imported into CorelDraw 12 for the construction of figures. As recommended by the guidelines of Rossner and Yamada [2004], these images were treated identically and underwent minimal manipulation in order to preserve the original data. The confocal images shown are representative of four animals from each experimental group.

Tissue Fractionation and Sample Preparation

Lysates for Western blotting and in vitro kinase assay were prepared by a modification of the method described by Klip et al. [1987]. Briefly, tissues were homogenized in four volumes of buffer (150 mM NaCl; 5 mM $MgCl_2$; 2 mM EGTA; 0.2 mM DTT; 10 mM MES, pH 6.5, 5 mM glucose, and Protease Inhibitor Cocktail III (Calbiochem)). The homogenates were centrifuged at 2,000*g* for 10 min to clear nuclei and large particles, and the postnuclear supernatant was centrifuged at 20,000*g* for 30 min, to pellet the myofibrillar fraction. The resulting supernatant was centrifuged at 150,000*g* for 1 h to pellet the crude membrane fraction. The final supernatant was reserved as the cytosolic fraction. The nature of these fractions was confirmed by Western blotting that revealed the relative enrichment of dystrophin in the crude membranes and the presence of desmin in the myofibrillar fractions of control animals. In some cases heart and brain tissue were homogenized in CaMKII extraction buffer [Abraham et al., 1996] and centrifuged at 100,000*g* to obtain the supernatant that was then considered the total CaMKII extract. Each sample fraction was assayed for protein content with the DC protein reagent kit (Bio-Rad) using bovine serum albumin as the standard and, either used immediately or stored at −80°C prior to use.

Electrophoresis and Western Blotting

This was performed essentially as described in Abraham et al. [1997]. Equal amounts of tissue extract proteins (10–50 μg) were separated on 10% SDS–polyacrylamide gels and transferred onto nitrocellulose membranes by Western blotting. The blots were blocked using 5% non-fat dry milk in a washing buffer of 10 mM Tris (pH 7.5), 100 mM NaCl, and 0.1% Tween 20. Affinity purified antibodies to proteins of interest were diluted in blocking buffer and incubated with the membranes for 1 h at 37°C and then rinsed in washing buffer six times over 30 min. Blots are then incubated with appropriate secondary antibodies coupled to HRP (1:1,000 dilution) and the immune complexes were detected using the ECL kit (Amersham Pharmacia) and exposure to X-ray film. The blots were reprobed with additional antibodies after 30 min of treatment with stripping buffer at 50°C (62.5 mM Tris, pH 6.7;

100 mM β-mercaptoethanol and 2% sodium dodecylsulfate). The Western blotting data shown in each figure is representative of 2–4 separate experiments.

CaMKII Activity Assay

Kinase activity in various muscle fractions was assayed as outlined previously [Abraham et al., 1996]. Total CaM kinase II activity in the various cell fractions was determined in a 25 μl reaction volume containing 10 mM MOPS (pH 7.4), 10 mM $MgCl_2$, 3 mM EGTA, 4 mM $CaCl_2$, 400 nM CaM, 0.2 mM [γ-^{32}P] ATP (400–1,000 cpm/pmol specific activity), 20 μM autocamtide-2 (specific substrate), and 0.5–2.0 μg of lysate proteins. Reactions were carried out at 30°C in a shaking incubator for 3 min and then terminated by precipitation of the phosphopeptide onto Whatman P81 paper. The papers were rinsed thoroughly in 75 mM phosphoric acid and the adherent radioactivity was quantified by liquid scintillation counting. Kinase activity is expressed as nmoles of phosphate transferred to the substrate per min per mg lysate protein. In each case the kinase activity data are the mean of two animal samples assayed in triplicate.

RESULTS

δCaMKII Expression in Regenerating Myofibers From Normal Mice

The expression of distinct isoforms of δCaMKII in embryonic cardiac myofibers and skeletal muscle led us to hypothesize that regenerating skeletal muscle would express high levels of these kinase isoforms. To obtain evidence to this effect, muscle necrosis and regeneration was induced in normal 4-week-old mice by intramuscular injection with 50% glycerol. Standard hematoxylin/eosin and propidium iodide staining allowed for localization of muscle fibers with centralized nuclei indicating that they had undergone regeneration (Fig. 1I). Three days after wounding with glycerol there were very few regenerating fibers, while 7-day treated quadriceps muscle showed regenerating myofibers around the edges of the necrotic zone. Additionally, confocal imaging revealed that these myofibers exhibited enhanced expression of desmin and δCaMKII when tissue sections were co-stained with anti-desmin and commercial δCaMKII antibodies (compare panels A–C with D–F, Fig. 1). The desmin stain confirmed the skeletal muscle nature of the regenerated fibers, while the increased expression of δCaMKII was corroborated by similar staining obtained with a δ-kinase specific antibody directed against a distinct site on δCaMKII (CK2-Delta, compare panels D and H, Fig. 1). The enhanced staining for δCaMKII was clearly seen in sections co-stained with propidium iodide that specifically labeled centralized nuclei (asterisk, panel H Fig. 1). The specificity of staining for δCaMKII in muscle fibers was demonstrated by the high degree of overlap produced by dual labeling with the two different CaMKII antibodies (not shown). It was also observed that apparently intact fibers (non-regenerating) close to the necrotic zone stained with lesser intensity to both δCaMKII antibodies than fibers that were found more distally or those from the control quadriceps. This loss in δCaMKII staining was apparent in intracellular and periplasmic regions of myofibers and is notable when comparing panel A versus D (arrowheads) or panel G versus H (arrowheads) of Figure 1. A similar loss in desmin stain was not observed in non-regenerating fibers of the peri-necrotic region of glycerol-treated quadriceps muscle (panel B vs. E, Fig. 1). Co-staining of longitudinal sections of muscle with anti-desmin and δCaMKII antibodies showed that a proportion of the kinase co-localizes with the intermediate filament at the Z-disk of muscle fibers (not shown).

Standard imaging after hematoxylin/eosin stain of sections from 7-day glycerol-treated quadriceps revealed spindled-shaped cells in the necrotic cavity close to the regenerating fibers (Fig. 1I). In contrast, fewer spindle-shaped cells were observed in the same region at 3 days after glycerol treatment (not shown). Higher resolution confocal imaging of these spindle-shaped cells showed that they also express significant amounts of desmin and δCaMKII as indicated by anti-desmin and CK2-Delta staining (asterisks, Fig. 2). In many cases these spindle-shaped fibers were found very close to smaller diameter myofibers in what appeared to be myoblasts fusing to form larger fibers (arrows, Figs. 1H and 2).

CaMKII Expression in Skeletal Muscle of the *mdx* Mouse

The lack of dystrophin in skeletal muscle fibers of *mdx* mice results in widespread necrosis followed by muscle regeneration that

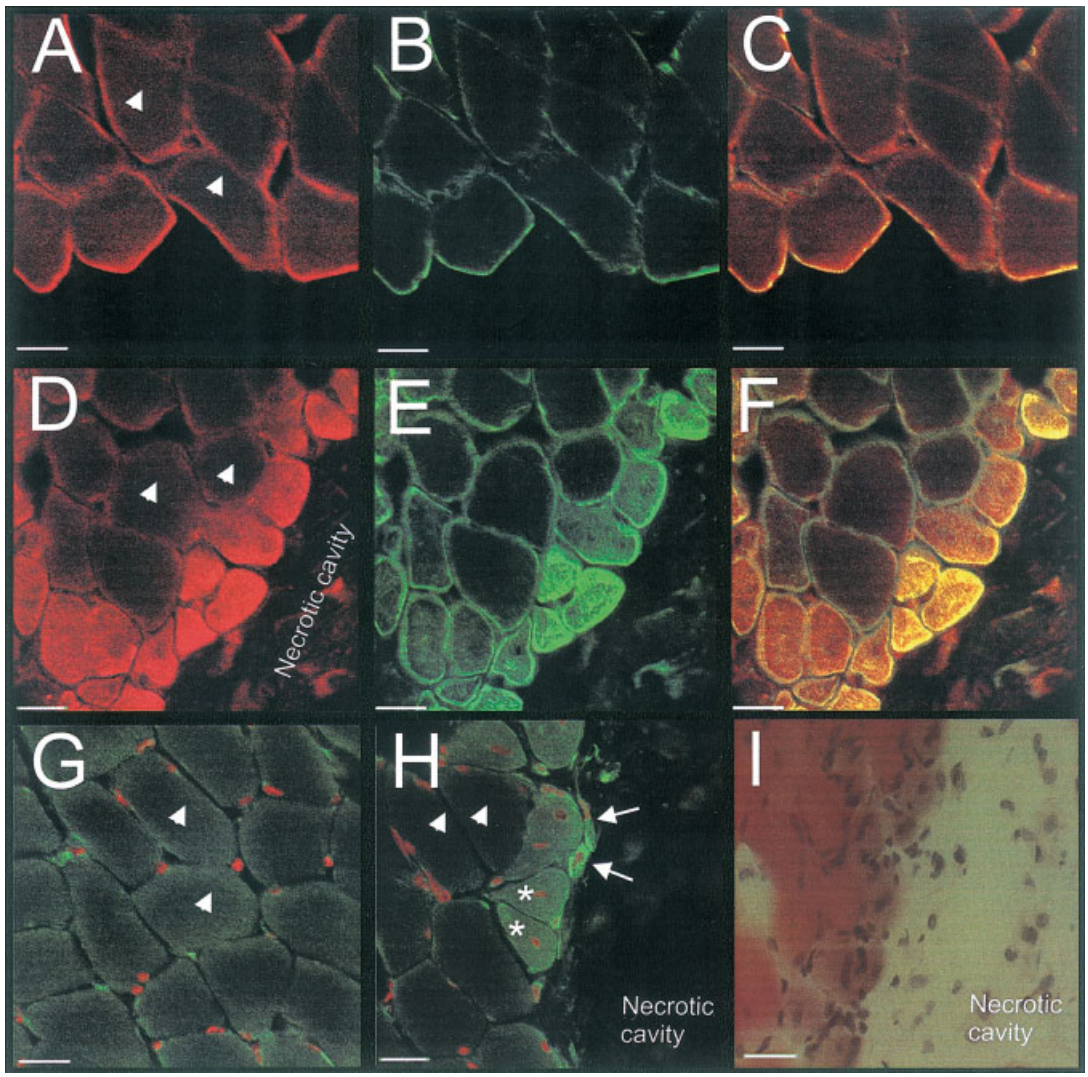

Fig. 1. δCaMKII expression in quadriceps muscle of control and glycerol-treated mice. Muscle cross-sections from control tissue co-stained with A-17 δCaMKII antibody (**A**), desmin antibody (**B**), and merged image (**C**). Cross-sections from glycerol-treated quadriceps co-stained with A-17 antibody (**D**), desmin antibody (**E**), and merged image (**F**). Control (**G**) and glycerol-treated (**H**) quadriceps co-stained with CK2-Delta antibody (green) and propidium iodide (red). Arrows indicate myoblasts fusing with regenerating myofibers. Arrowheads comparing δCaMKII expression (panel A vs. D and panel G vs. H). Scale bar = 20 μm. **I**: Hematoxylin/eosin stain of adjacent section to (H) imaged with 40 × objective.

peaks at about 4 weeks of age. Four to six-week old *mdx* mice were used to determine if expression of δCaMKII isoforms was altered during the post-necrotic regeneration period. Due to the extent of regeneration that occurs in *mdx* mice at this age it was hypothesized that δCaMKII expression would be elevated and could be detected by Western blotting. Immunoblotting of hind limb preparations with the δ isoform specific CK2-Delta antibody revealed two immunoreactive bands localized primarily in the myofibrillar and membrane fractions of skeletal muscle fibers (Fig. 3A). The relative molecular size of these kinase isoforms was estimated to be 56 and 58 kDa, which appear consistent with the δ_9 and δ_4 CaMKII isoforms [respectively, Schworer et al., 1993; Hagemann et al., 1999]. However, contrary to our expectations these immunoblots also demonstrated that the level of δCaMKII in the myofibrillar and membrane fractions of dystrophic hind limbs was reduced when compared to those from age-matched control mice (Fig. 3A). Additionally, the decreases in δCaMKII levels were found to be sustained in *mdx* mice as old as 6 months (not shown).

In vitro kinase assays demonstrated that total CaMKII activity in myofibrillar fractions

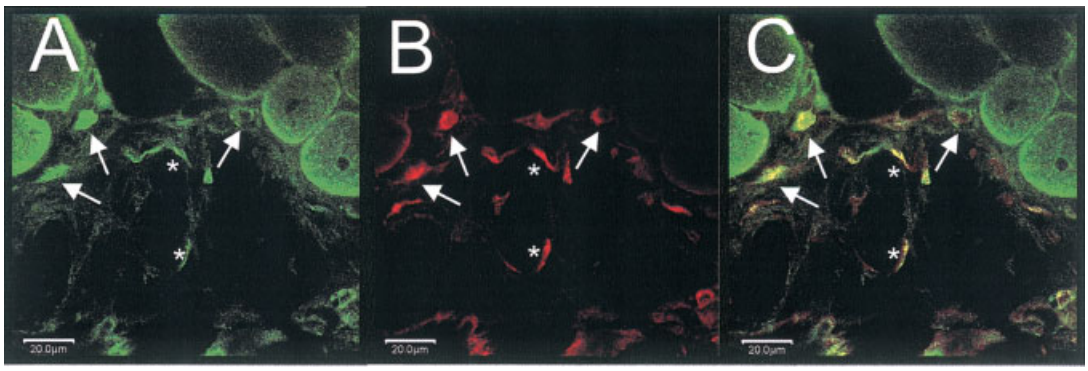

Fig. 2. Confocal images of myoblasts in the necrotic cavity of glycerol-treated quadriceps muscle. Muscle cross-sections co-stained with CK2-Delta (**A**) or desmin (**B**) antibodies and the merged image (**C**). Asterisks: replicating myoblasts in the necrotic cavity; arrows: myoblasts fusing to form new myofibers. Calibration bar = 20 µm.

from *mdx* leg muscles was reduced by 50% when compared to control fractions (one-way ANOVA, $P < 0.05$); however, activities in the other fractions were not significantly different between *mdx* and control animals (Fig. 3B). Confocal microscopy was used to determine if regenerating myofibers could be identified in *mdx* tissue, and if these fibers displayed altered δCaMKII expression. Staining of quadriceps and tibialis muscle of 4–6 week old *mdx* mice with δCaMKII antibody revealed that overall kinase expression in dystrophic hind limbs was less than that seen in control tissues (Fig. 4). However, both quadriceps and tibialis muscle from *mdx* mice contained groups of myofibers with both centralized nuclei (determined by propidium iodide stain) and enhanced δCaMKII expression (Fig. 4). This differential staining in regenerating and non-regenerating fibers gave a patchy bright stain that was made prominent by the low staining of the surrounding fibers. As seen in the regenerating fibers after glycerol treatment, this elevated expression of δCaMKII was seen throughout the cross-section of myofibers with particular concentration in the sub-sarcolemmal space (compare panel D, Fig. 1 with panel B, Fig. 4). δCaMKII was also localized to the perinuclear region of myofibers and the expression in this region appeared to be increased in dystrophic tissue as compared to control fibers (arrows, Fig. 4).

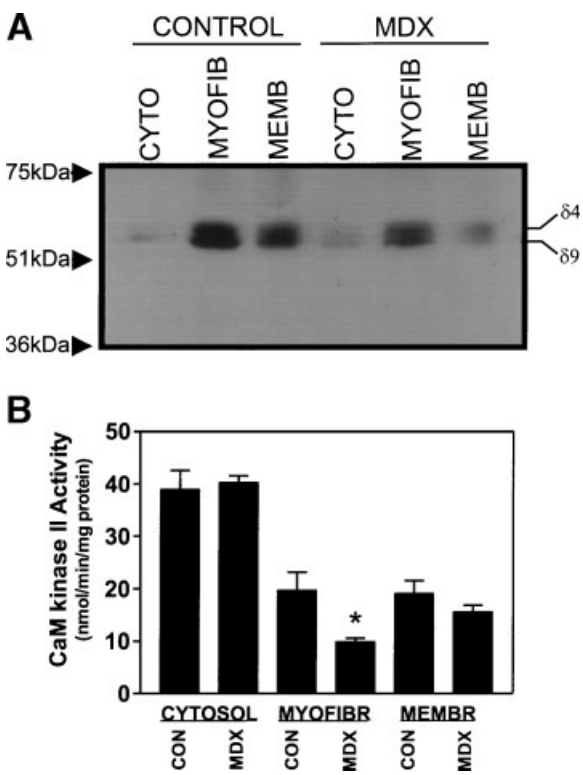

Fig. 3. CaMKII content in the hind limbs of 4–6 week old *mdx* and control mice. **A**: Western blot of subcellular fractions of normal and *mdx* muscle probed with δCaMKII-specific antibody (CK2-Delta). **B**: In vitro CaMKII activity in subcellular fractions from control and *mdx* hind limbs; bars represent mean ± SD of two animals in each group assayed in triplicate. Myofibr, myofibrillar; membr, crude membrane fractions. (*) Significantly different from control myofibrillar activity ($P < 0.05$).

The expression of δCaMKII in brain and heart tissue was also investigated to determine if decreased kinase content seen in hind limb muscles was a global phenomenon in the *mdx* mouse. Immunoblotting of heart extracts with the δ-specific antibody revealed that these tissues expressed 54 and 55 kDa isoforms of δCaMKII, while brain extracts contained 54, 56, and 60 kDa isoforms of the kinase.

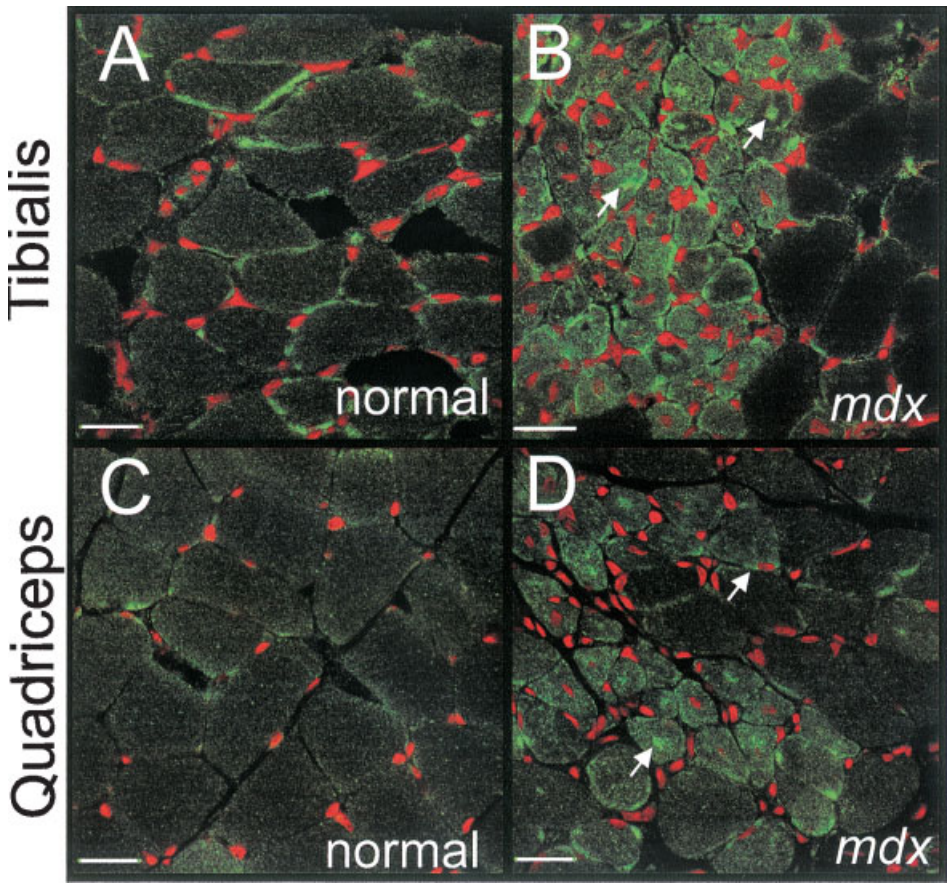

Fig. 4. δCaMKII expression in tibialis and quadriceps muscle from 4–6 week old control and *mdx* mice. Confocal images of tibialis muscle from control (**A**) and *mdx* (**B**) mice co-stained with δCaMKII antibody (green) and propidium iodide (red). Confocal images of quadriceps muscle from control (**C**) and *mdx* (**D**) mice co-stained with δCaMKII antibody (green) and propidium iodide (red). Arrows: perinuclear δCaMKII staining. Calibration bar = 20 μm.

Immunoblotting equivalent amounts of total cardiac or brain kinase extracts from *mdx* and control mice revealed that δCaMKII content in these tissues was not altered due to dystrophic disease (Fig. 5A). In vitro kinase assays of subcellular fractions from cardiac and brain tissue also demonstrated that overall CaMKII activity in these tissues was not altered by the lack of dystrophin (Fig. 5B,C).

DISCUSSION

To investigate the role of δCaMKII in skeletal muscle regeneration an experimental model of intramuscular glycerol injections was utilized to produce necrosis and regeneration. Such treatment has been shown to produce cell membrane disruption, loss of myofibrils, myonecrosis, and muscle regeneration [Kawai et al., 1990]. The quadriceps of normal mice were followed for up to 7 days after intramuscular administration of 50% glycerol, and regenerating myofibers were identified by the presence of centralized nuclei [Kawai et al., 1990; Boland et al., 1995; Hoffman, 2001]. Immunostaining with specific antibodies to desmin and δCaMKII followed by confocal imaging showed that regenerating fibers express higher levels of both proteins than non-regenerating myofibers. The increased levels of desmin in muscle fibers with centralized nuclei is consistent with previous reports that showed that desmin expression is upregulated in myoblasts [Kaufman and Foster, 1988; Rantanen et al., 1995] as well as regenerating myofibers [Agubulut et al., 2001; Vaittinen et al., 2001]. The upregulation of δCaMKII in regenerating myofibers was confirmed by confocal imaging using two different antibodies directed against distinct epitopes on the kinase. The CK2-Delta antibody recognizes the extreme C-terminus unique to δCaMK isoforms [Schworer et al., 1993; Van Riper

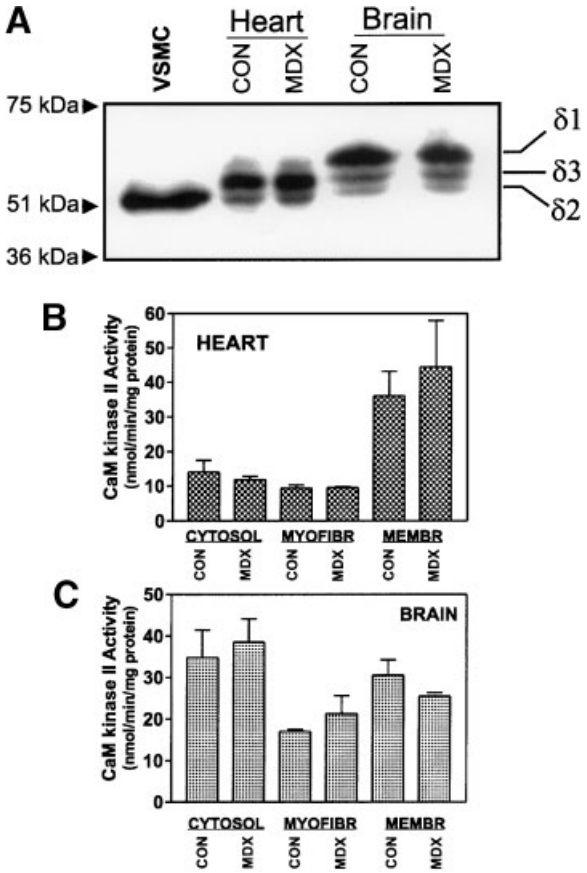

Fig. 5. CaMKII content in heart and brain extracts of 4–6 week old *mdx* and control mice. **A**: Western blot of 30 μg total extracts from heart and brain tissues probed with δCaMKII-specific antibody. VSMC = equivalent amount of cultured vascular smooth muscle cell extract included for identification of δ_2-isoform. In vitro CaMKII activity in subcellular fractions of heart (**B**) and brain (**C**) tissues. Data are mean ± SD of two animals in each group assayed in triplicate. Myofibr, myofibrillar; membr., crude membrane fractions.

et al., 2000] while the commercial A-17 antibody is directed against an internal region of the kinase with apparently little cross-reactivity to other CaMKII isoforms (datasheet, Santa Cruz). Co-staining of post-necrotic regions with both δCaMKII antibodies demonstrated virtually overlapping staining as determined by co-localization analysis software. This could be accomplished since the antibodies were from two different species (rabbit or goat) and could be independently detected by appropriately labeled secondary antibodies. In our view, the strategy of using two distinct antibodies to define the specificity of immunofluorescent staining is superior to the use of excess amounts of competing immunogen which could sequester the antibody away from both specific and non-specific sites. The results of this study clearly demonstrate that δCaMKII expression is elevated, in parallel with desmin, in regenerating myofibers from normal mice. Closer examination of these tissues also revealed mononuclear spindle-shaped cells in proximity to the regenerating fibers displaying remarkable staining for δCaMKII. In some cases these cells could be seen in between regenerating fibers or fusing with small diameter myofibers suggesting that they are myoblasts. Specific staining of these cells with anti-desmin antibody verified that they were myoblasts that appear to be participating in the muscle repair process [Kaufman and Foster, 1988; Saito and Nonaka, 1994; Rantanen et al., 1995]. Thus it would appear that both myoblasts and regenerating myofibers express high levels of δCaMKII in a manner analogous to desmin.

The lack of functional dystrophin in the *mdx* mouse results in widespread skeletal muscle necrosis that peaks about 4 weeks after birth [Cooper, 2001]. Significant muscle regeneration and repair follows this event and this genetic model was used to determine if δCaMKII expression was altered in dystrophic pathology. Thus our studies focused on the expression of δCaMKII isoforms in wild-type and *mdx* mice 4–6 weeks after birth. Immunoblotting extracts from the hind limbs of *mdx* and control mice with a δCaMKII-specific antibody identified two proteins of 56 and 58 kDa molecular weight. These proteins most likely correspond to the δ_9 and δ_4 isoforms (respectively) of CaMKII that have been identified previously [Schworer et al., 1993; Hagemann et al., 1999; Hoch et al., 2000]. Western blotting of heart extracts from control and mdx mice revealed the expression of 54 and 55 kDa immunoreactive bands most likely corresponding to the δ_2 and δ_3 isoforms of CaMKII [Schworer et al., 1993; Hagemann et al., 1999]. Brain extracts contained similar sized δCaMKII isoforms as well as a 60-kDa form that is consistent with the δ_1 isoform [Braun and Schulman, 1995]. These studies taken together confirm earlier findings that skeletal muscle cells express unique splice-variants of δCaMKII [Schworer et al., 1993; Hagemann et al., 1999; Hoch et al., 2000].

We hypothesized that the marked regeneration of skeletal muscle in *mdx* mice would lead to enhanced δCaMKII expression in this tissue. However Western blotting of total hind limb extracts indicated that *mdx* muscles experience

a loss in δCaMKII expression that is sustained in these mice for a period of at least 6 months. This decrease in δCaMKII expression was not seen in heart and brain extracts from the *mdx* mouse when compared to their age-matched controls. The immunoblotting data were supported by in vitro activity assays that showed that total CaMKII content in the myofibrillar fractions from hind limbs of *mdx* mice was significantly reduced when compared to control tissues. In contrast these same assays demonstrated that CaMKII content in the various fractions of cardiac and brain tissues from *mdx* mice were unchanged.

Even though some discrepancies in CaMKII content were evident when measured by Western blotting versus in vitro activity assays (compare cytosolic activity and δCaMKII content; Fig. 3A,B) both methods demonstrated similar trends in CaMKII expression in dystrophic tissue. The specificity of the in vitro CaMKII activity assay has been established by previous studies [Abraham et al., 1996], but unlike Western blotting it is unable to distinguish between the various kinase isoforms found in skeletal muscle [e.g., δ_4, β_M, and γ_B; Schworer et al., 1993; Bayer et al., 1998]. The inability of the in vitro activity assay to detect decreases in CaMKII in the membrane fractions of *mdx* hind limb muscle (compare Fig. 3A,B) may be due to other CaMKII isoforms (e.g., γ_B, β_M) not being downregulated in dystrophic tissue. It is possible that other CaMKII isoforms are actually upregulated in dystrophic skeletal muscle, however this has not been established and is the subject of future studies.

The mechanism for decreased δCaMKII expression in hind limb muscle of mdx mice is unknown but appears to be restricted to skeletal muscle tissue. One explanation for this finding may be that cardiac and neuronal tissue in these animals do not undergo cell death to the same extent observed in skeletal muscle [Hoffman, 2001]. Therefore, the observed downregulation of δCaMKII may be the result of enhanced proteolytic cleavage in dystrophic tissue [Alderton and Steinhardt, 2000]. This is supported by our observations that even quadriceps muscle (from normal mice) damaged by glycerol treatment showed evidence of decreased δCaMKII content in myofibers close to the necrotic zone (Fig. 1D,H). Further, in vitro studies have described the proteolytic processing of CaMKII to active and inactive fragments by serine proteases such as calpain [Kwiatkowski and King, 1989]. Nonetheless, we cannot rule out the possibility that δCaMKII content is decreased in the *mdx* muscle fibers due to diminished transcription or translation of kinase mRNA. The ability of regenerating myofibers in *mdx* mice to express high levels of the kinase would argue against an inherent block of δ kinase gene expression in these animals.

As seen in the regenerating myofibers after glycerol-injury, enhanced δCaMKII expression was seen in *mdx* hind limb muscle that had undergone regeneration. These fibers could be identified by their smaller diameter and centralized nuclei [Torres and Duchen, 1987; Coulton et al., 1988; Cooper, 2001]. Qualitatively there was no difference in the localization of δCaMKII in regenerating fibers from either the glycerol-treated or the *mdx* mice. In both cases increased δCaMKII expression could be seen throughout the cytoplasmic/myofibrillar region as well on the sarcolemmal and perinuclear regions.

In addition to altered expression of δCaMKII in muscle pathology, this study also establishes the unique localization of this kinase in skeletal muscle. Confocal imaging suggests that δCaMKII isoforms are located in the cytoplasmic/myofibrillar and membrane fractions of muscle cells; however, subcellular fractionation and Western blotting indicates that there is a relative enrichment of the kinase in the myofibrillar and membrane fractions of the cell. The apparent discrepancy between these results may be due to the inability of microscopy to distinguish between the cytoplasmic and myofibrillar compartments in these conditions. Nonetheless the clear plasma membrane and sub-membrane localization of δCaMKII can be deduced from both the imaging and Western blotting studies. Kinase activity assays confirm that CaMKII is located in the myofibrillar and membrane fractions as well as in the cytoplasmic pool of skeletal muscle. As mentioned earlier the bulk of the kinase contributing to the cytoplasmic activity may not be δ-isoforms but rather γ- or β-isoforms that has been previously described [Schworer et al., 1993; Bayer et al., 1998]. The localization of δCaMKII in the sarcolemmal fraction of skeletal fibers is consistent with reports of a CaM kinase activity associated with the junctional sarcoplasmic reticulum [Chu et al., 1990], and the dystrophin-glycoprotein complex [Madhavan and Jarrett, 1994]. The myofibrillar location of the kinase is supported

by the finding that CaM kinase activity co-purifies with various cytoskeletal elements [Bahler and Greengard, 1987; Yano et al., 1994; McNeil and Colbran, 1995; Ogawara et al., 1995]. In addition the present study demonstrates that δCaMKII co-localizes with the intermediate filaments at the Z-disk [Paulin and Li, 2004], as well as in the perinuclear region of myofibers. Using Western blotting, kinase activity assays and confocal microscopy, the current study clearly establishes the localization of δCaMKII in discrete regions of murine skeletal muscle.

The picture that emerges from these studies is that δCaMKII expression in replicating myoblasts and newly formed myofibers is closely linked to the overall process of skeletal muscle repair. The precise functions of CaM kinases in muscle regeneration remain undetermined but work in cardiac and neuronal development would support a prominent role for these enzymes. The expression of δ_4CaMKII in adult skeletal muscle is well documented [Schworer et al., 1993; Hagemann et al., 1999] but this isoform only appears in embryonic and neonatal cardiac myocytes and is rapidly down-regulated thereafter [Hagemann et al., 1999]. Cultures of embryonic neurons and the tissues of the developing nervous system apparently express δ and γ CaMKII isoforms much earlier than the predominant α and β isoforms that are seen in adult tissue [Bayer et al., 1999; Donai et al., 2000]. Thus the continued expression of "embryonic" δCaMKII isoforms in adult skeletal muscle may be central to its considerable regenerative capacity. A number of investigators have demonstrated that CaM kinases can regulate the expression of muscle-related genes as well as control myoblast fusion and myotube formation in culture [Scicchitano et al., 2002, 2005; Xu et al., 2002; Linseman et al., 2003]. These studies either used pharmacological inhibitors or over-expression strategies to demonstrate the potential role of CaM kinases in muscle function but did not identify the in vivo kinase(s) responsible for these actions. Our study complements these previous findings and extends them by demonstrating that δ-isoforms of CaMKII are upregulated during the muscle repair process in vivo. Conversely, CaMKII inhibition appears to cause apoptosis in different cell types (Tombes et al., 1995; Linseman et al., 2003; Abraham, unpublished in vascular smooth muscle). The latter observations are particularly relevant if one considers the evidence that apoptosis precedes necrosis in the skeletal muscle of the *mdx* mouse [Tidball et al., 1995; Sandri et al., 1998]. These reports combined with our current observation, of decreased δCaMKII expression in normal fibers from the peri-necrotic regions of glycerol-treated limbs or non-regenerating fibers from *mdx* muscle, raises the intriguing possibility that the loss of δ kinases contributes to the destruction of muscle fibers. Thus one or more δCaMKII isoforms may function as cell survival factors in skeletal muscle. Such a role for CaMKII has been hypothesized by Rando [2001] but remains to be demonstrated experimentally.

In summary, our study is the first to report that expression of δCaMKII isoforms is enhanced in regenerating fibers from the hind limbs of glycerol-injured normal mice or the *mdx* mouse. Additionally, our study demonstrates that basal δCaMKII levels in apparently intact muscle fibers from mdx mice are chronically depressed. This decrease in kinase expression appears to be long lasting and specific to skeletal muscle. To our knowledge this is the first report demonstrating that δCaMKII expression may be differentially regulated in skeletal muscle fibers undergoing regeneration and perhaps necrosis as well. We propose that δCaMKII expression is critical for the regeneration of myofibers during muscle repair and may also behave as a survival factor. Establishment of such a dual role for δCaMKII isoforms in skeletal muscle requires further work and would contribute to our understanding of muscle development and pathology.

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