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CaMKII protein expression and phosphorylation in human skeletal muscle by immunoblotting: Isoform specificity

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ABSTRACT

Calcium (Ca^{2+})/calmodulin-dependent protein kinase II (CaMKII) is activated during exercise by reactive oxygen species (ROS) and Ca^{2+} transients initiating muscle contraction. CaMKII modulates antioxidant, inflammatory, metabolic and autophagy signalling pathways. CaMKII is coded by four homologous genes (α, β, γ, and δ). In rat skeletal muscle, δ_D, δ_A, γ_D, γ_B and β_M have been described while different characterisations of human skeletal muscle CaMKII isoforms have been documented. Precisely discerning between the various isoforms is pivotal for understanding their distinctive functions and regulatory mechanisms in response to exercise and other stimuli. This study aimed to optimize the detection of the different CaMKII isoforms by western blotting using eight different CaMKII commercial antibodies in human skeletal muscle. Exercise-induced posttranslational modifications, i.e. phosphorylation and oxidations, allowed the identification of specific bands by multitargeting them with different antibodies after stripping and reprobing. The methodology proposed has confirmed the molecular weight of β_M CaMKII and allows distinguishing between γ/δ and δ_D CaMKII isoforms. The corresponding molecular weight for the CaMKII isoforms resolved were: δ_D, at 54.2 ± 2.1 kDa; γ/δ, at 59.0 ± 1.2 kDa and 61.6 ± 1.3 kDa; and β_M isoform, at 76.0 ± 1.8 kDa. Some tested antibodies showed high specificity for the δ_D, the most responsive isoform to ROS and intracellular Ca^{2+} transients in human skeletal muscle, while others, despite the commercial claims, failed to show such specificity.

1. Introduction

Calcium (Ca^{2+})/calmodulin-dependent protein kinase II (CaMKII) is activated during exercise by reactive oxygen species (ROS) [1] and the Ca^{2+} transients triggering muscle contraction [2]. CaMKII modulates the antioxidant signalling pathways mediated by Keap1, NRF2 and NF-κB [3–5] and is implicated in the regulation of glycolytic enzymes [6], glycogen synthesis [7], glucose transport [8,9], protein synthesis and ion concentrations [10,11], muscle contraction and relaxation [10], nitric oxide synthase [12], and autophagy [13], among other functions. Besides, CaMKII is instrumental in the muscle adaptive response to regular exercise and is a chief determinant of muscle phenotype [14,15]. For these reasons, studying CaMKII is fundamental for a mechanistic approach in skeletal muscle physiology research, explaining the growing

Abbreviations: CaMKII, Calcium (Ca^{2+})/calmodulin-dependent protein kinase II; HDAC4, Histone deacetylase 4; Keap1, Kelch-like ECH-associated protein 1; MHC-IIx, Myosin heavy chain isoform IIX; MW, molecular weight; NF-κB, Nuclear factor kappa B; NRF2, Nuclear factor erythroid 2-related factor 2; ROS, Reactive oxygen species; RT, Room temperature; TBS-T, Tris-buffered saline containing 0.1 % Tween 20; VL, *Vastus lateralis*.

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interest in better characterising this enzyme.

CaMKII is a multimeric enzyme encoded by four homologous genes (α, β, γ, and δ) [16,17]. Each isoform comprises a catalytic domain, a variable domain, and an oligomerisation domain. The N-terminal catalytic domain features a CaM-binding site housing the stimulatory autophosphorylation site (Thr^{287}) and the inhibitory phosphorylation site (Thr^{306}). The central variable domain, susceptible to alternative splicing, contributes to the notable diversity within this kinase family [18]. Despite high sequence homology among the four genes [19], indicating conservation of essential functions, the holoisoforms exhibit differential tissue expression [20,21] and exist as several alternative spliced variants. The β isoform is neuron-specific, and its spliced variant $β_M$ is present in skeletal muscle [22]. The δ and γ isoforms are ubiquitous [22], the δ being predominant in cardiac myocytes [23–25] and the γ prevalent in smooth muscle [26–28]. The α isoform (54.0 kDa) is expressed primarily in the brain [29].

The presence of β, γ, and δ CaMKII isoforms in skeletal muscle has been revealed mainly by Western blot with isoform-specific antibodies [30–35]. In rodent skeletal muscle, the isoforms $β_M$, $γ_B$, $δ_D$, and $δ_A$ have been described [30,36]. In human skeletal muscle, some researchers did not differentiate between γ and δ, reporting the two variants of the δ and the γ isoforms conjointly while identifying the $β_M$ isoform separately [4,34,35,37,38]. Interestingly, a specific regulation of some of these isoforms has been reported after exercise training. For example, Thomassen et al. [39] reported upregulation of $pThr^{287}$- γ/δ CaMKII and $pThr^{287}$-$β_M$ CaMKII, as well as an increased amount of the total protein expression of γ/δ CaMKII after a 7-week high-intensity training in cyclists. Likewise, Popov et al. [40] reported increased basal $pThr^{287}$-CaMKII protein expression without distinguishing between isoforms after aerobic training in untrained men. The latter may be due to the difficulty of separating and distinguishing between the different isoform bands in Western blot analyses from human skeletal muscle [3–5,14,34,35,37–42]. Correctly identifying the CaMKII isoforms in human skeletal muscle, particularly the δ isoform [14], is crucial for better understanding their specific functions and regulation in response to exercise and other stimuli.

Therefore, the purpose of this study was to develop a protocol to optimize the identification of the $β_M$, γ/δ and $δ_D$ CaMKII variants and their respective activating phosphorylation (Thr^{287}) and oxidation ($Met^{281/282}$) using eight different commercial antibodies through immunoblotting in human skeletal muscle.

2. Materials and methods

2.1. General overview

This study is based on the analysis of resting and post-exercise skeletal muscle biopsies obtained in research projects from our laboratory to determine mechanisms of fatigue after a strength-training intervention [14,43] and high-intensity exercise [3,4,44]. In the main experiments 1 and 2, resting muscle biopsies were obtained from the *vastus lateralis* (VL) muscle of a healthy, physically active human. Experiment 3 included twenty-two physically active young male participants from whom VL muscle biopsies were taken before and after a strength-training program [14]. Biopsies corresponding to three participants were randomly chosen for the current determinations. In experiment 4, eleven young active males had VL muscle biopsies taken at rest and immediately after a bout of intense exercise [3,4]. The biopsies pertaining to one of the participants were included in the present analyses. These experiments showed that CaMKII positively correlates with muscle hypertrophy and the total number of repetitions executed and negatively with the changes in MHC-IIx induced by the training program [14], and support a ROS-mediated CaMKII Thr^{287} phosphorylation during high-intensity exercise [3].

2.2. Muscle biopsies

Resting muscle biopsies were obtained after a 10-min rest in the supine position. Post-exercise muscle biopsies were obtained immediately after the exercise, with the subject seated on a cycle ergometer and leaning backwards, supported by a helper. The skin over the middle portion of the VL muscle was anaesthetised with 2 % lidocaine (2 mL) without adrenaline and injected above the superficial fascia, i.e., avoiding infiltrating the muscle belly. Following a 15-min interval, a 5-mm incision of the skin and superficial fascia was performed. Subsequently, a Bergstrom's biopsy needle was introduced through the incisions, and a muscle biopsy was obtained 2 cm below the fascia. The same medical doctor performed all biopsy procedures, ensuring standardisation. Complete information regarding participants' physical characteristics can be found in previous publications [3,4,14].

2.3. Muscle homogenates

Human skeletal muscle whole tissue lysates were prepared with ~10 mg of tissue ground by stainless steel balls for 1 min at 22 Hz in a Mikro-Dismembrator S (Sartorius, Goettingen, Germany) and immediately homogenised in urea lysis buffer (6 M urea, 1 % SDS, supplemented with 50X cOmplete protease and 10X PhosSTOP phosphatase inhibitor cocktails). Then, the lysates were centrifuged for 12 min at 25,200 g at 16 °C. The resulting supernatants were quantified in triplicate using the bicinchoninic acid assay [45]. The homogenised volumes were adjusted to obtain a concentration ranging from 5 to 6.5 μg/μL in experiments 1 and 4, 0.4 μg/μL and 5.5 μg/μL in experiment 2, and ~2.8 μg/μL in experiment 3. For experiments 1 and 2, 0.5–22.5 μg of the same lysate was loaded and electrophoresed. To ensure the reproducibility of the different antibodies and to provide sufficient area for proper incubation, the same lysate was loaded in duplicate for each antibody tested. After trying different protein amounts, the protein quantities were chosen to allow the visualisation of most CaMKII isoforms within a singular chemiluminescent exposure employing the antibody conditions defined in the following lines. For experiment 2, the anti-CaMKII total (no. 611292, BD Biosciences) was used as a reference antibody to be compared with the isoform-specific antibodies.

For experiments 3 and 4, 10–22.5 μg were loaded for electrophoresis. The amount of protein loaded was optimised by determining the range of protein loading for which the antigen-antibody response was linear, generally ranging from 5 to 30 μg, considering potential changes in protein expression levels [3,4,14]. Experiments 3 and 4 were conducted as additional assays to validate the banding patterns observed in experiments 1 and 2. Given the staining consistency and relatively low variability observed between the duplicates in experiments 1 and 2, running experiments 3 and 4 in duplicate was not deemed necessary.

2.4. Western blot

The samples were diluted one-third (v/v) in Laemmli reducing buffer (160 mM Tris-HCl, pH 6.8, 5.9 % SDS, 25.5 % glycerol, 15 % β-mercaptoethanol-bromophenol blue) and electrophoresed at 4 °C for 50 min at 80V to stack the samples and 90 min at 170V in 10 % hand-cast gels. After SDS-PAGE electrophoresis, the proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane (0.45 μm pores, Bio-Rad Laboratories, Hercules, CA, USA) with a Trans-Blot Cell (Bio-Rad) chamber for 90 min at 4 °C and 0.4 Å. For the accurate assessment of assay variability and to ensure optimal loading, transfer efficiency, and band separation, all membranes underwent staining using Reactive Brown 10 (Sigma Aldrich, St. Louis, MO, USA and Santa Cruz Biotechnology, Dallas, TX, USA) or Ponceau S (Sigma Aldrich, St. Louis, MO, USA). The membranes were blocked for 1 h at RT in 4 % bovine serum albumin (BSA) or 5 % skimmed milk-blocking buffer diluted in Tris-buffered saline containing 0.1 % Tween 20 (TBS-T) and incubated overnight for 12–15 h at 4 °C with primary antibodies. The primary

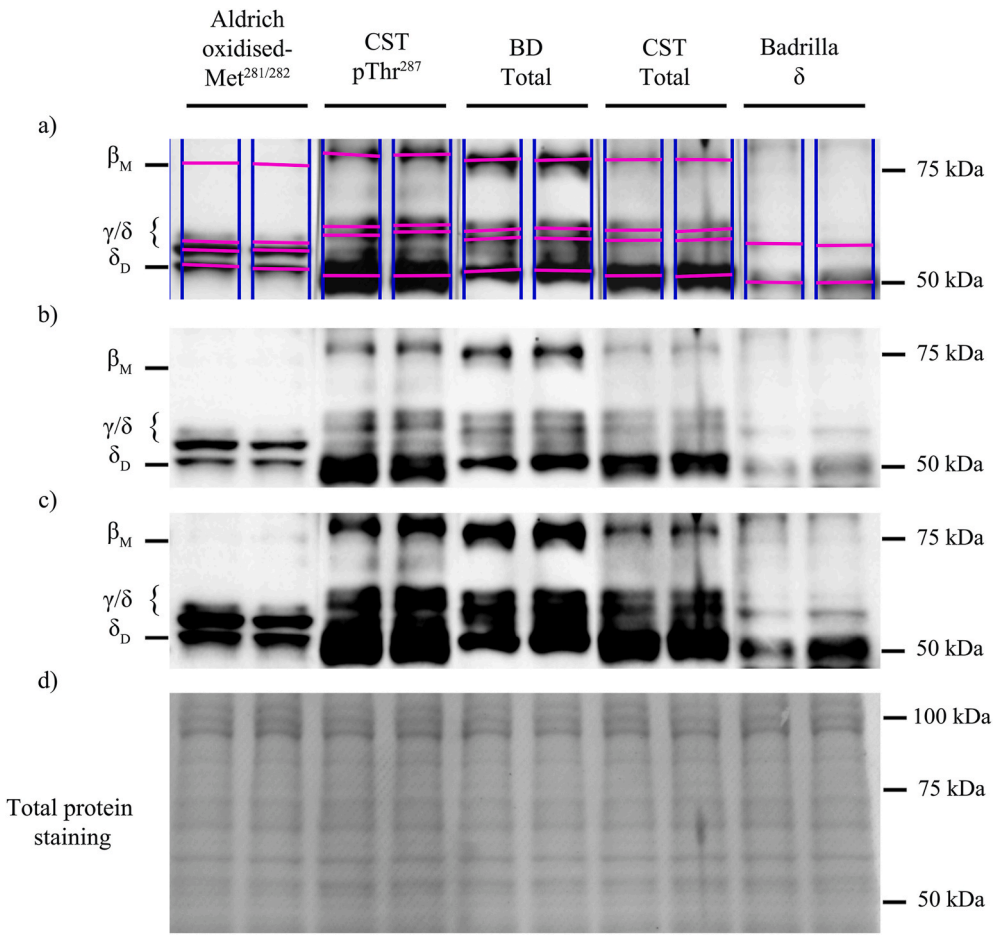

Fig. 1. CaMKII isoforms identification through immunoblotting in human skeletal muscle from a healthy participant obtained under resting conditions. **a) Representative image** obtained using Image Lab© software 6.0.1 (Bio-Rad) employing Lanes and Bands and MW Analysis Tools. The continuous purple line denotes the selected band used to calculate the molecular weight. Blue vertical lines delimit each lane. **b) Representative immunoblot** employing five commercial antibodies: Aldrich oxidised-Met$^{281/282}$, CST pThr^{287}, BD Total, CST Total, Badrilla δ. **c) Over-exposed immunoblot** targeting β_M with the Aldrich oxidised-Met$^{281/282}$ antibody. **d) Total protein staining** using Ponceau S. Isoforms (β_M, γ/δ and δ_D) are depicted on the left side of the panel. The band migration markers indicate the molecular weight in kDa.
Aldrich oxidised-Met$^{281/282}$: anti-oxidised-Met$^{281/282}$-CaMKII (no. 07–1387, Sigma Aldrich); CST pThr^{287}: anti-phospho-CaMKII-Thr^{287} (no. 12716, Cell Signaling Technology), BD Total: anti-CaMKII total (no. 611292, BD Biosciences), CST Total: anti-CaMKII total (no. 4436, Cell Signaling Technology), Badrilla δ: anti-CaMKII delta isoform (no. A010-55AP, Badrilla).

antibodies were anti-oxidised-Met$^{281/282}$-CaMKII diluted 1:5,000 in 5 % skimmed milk (no. 07–1387, Sigma Aldrich), anti-phospho-CaMKII-Thr^{287} diluted 1:2,000 in 4 % BSA (no. 12716, Cell Signaling Technology), anti-CaMKII total diluted 1:3,000 in 5 % skimmed milk (no. 611292, BD Biosciences), anti-CaMKII total diluted 1:2,000 in 4 % BSA (no. 4436, Cell Signaling Technology), anti-CaMKII delta isoform diluted 1:2,000 in 4 % BSA (no. 181052, Abcam), anti-CaMKII delta isoform diluted 1:2,500 in 4 % BSA (no. A010-55AP, Badrilla), anti-CaMKII gamma isoform diluted 1:2,000 in 4 % BSA (no. 12666-2-AP, Proteintech), and anti-CaMKII beta isoform diluted 1:2,000 in 4 % BSA (no. 376828, Santa Cruz Biotechnology). After the primary antibody incubation, the membranes were washed thrice for 10 min each with TBS-T. Subsequently, incubation with HRP-conjugated anti-rabbit (no. 111-035-144, Jackson ImmunoResearch; SC2004, Santa Cruz Biotechnology) or anti-mouse antibody (no. 115-035-003, Jackson ImmunoResearch; SC2031, Santa Cruz Biotechnology) was performed, with a dilution range of 1:5,000–1:50,000 in 5 and 2.5 % skimmed milk blocking buffers for all instances. Following incubation, chemiluminescent visualisation was conducted using Clarity™ Western ECL Substrate (Bio-Rad) and subsequent visualisation using a ChemiDoc™ Touch Imaging System (Bio-Rad). Pre-stained protein standards obtained from Bio-Rad (catalogue reference: 1610373, Bio-Rad) were captured under white light immediately after chemiluminescent imaging, with the membranes maintained in the same position.

2.5. Identification of CaMKII isoforms: Image Lab© software 6.0.1

The identification of CaMKII isoforms was based on their predicted electrophoretic size mobility (β_M: 72.7 kDa; δ_A: 60.0 kDa; γ_C: 56.0; γ_B:58.4 kDa; and δ_D: 58.4 kDa) [19] and the information provided in the antibody datasheet. Experimental molecular weights (MW) of each band resulting from the reactivity with antibodies targeting all total CaMKII and all phosphorylated isoforms, the specific antibodies for the β, γ, δ isoforms, and an antibody specific for oxidised methionine 281/282 were determined using the Lanes and Bands and MW Analysis Tools within the Image Lab© software 6.0.1 (Bio-Rad).

2.6. Identification of CaMKII isoforms: membrane stripping

To accurately identify CaMKII isoforms, the membrane in experiment 3, which contained the before and after strength training intervention samples from 3 subjects, and the membrane in experiment 4, which contained the resting and after acute exercise samples from 1 subject, underwent a stripping procedure and subsequent reprobing

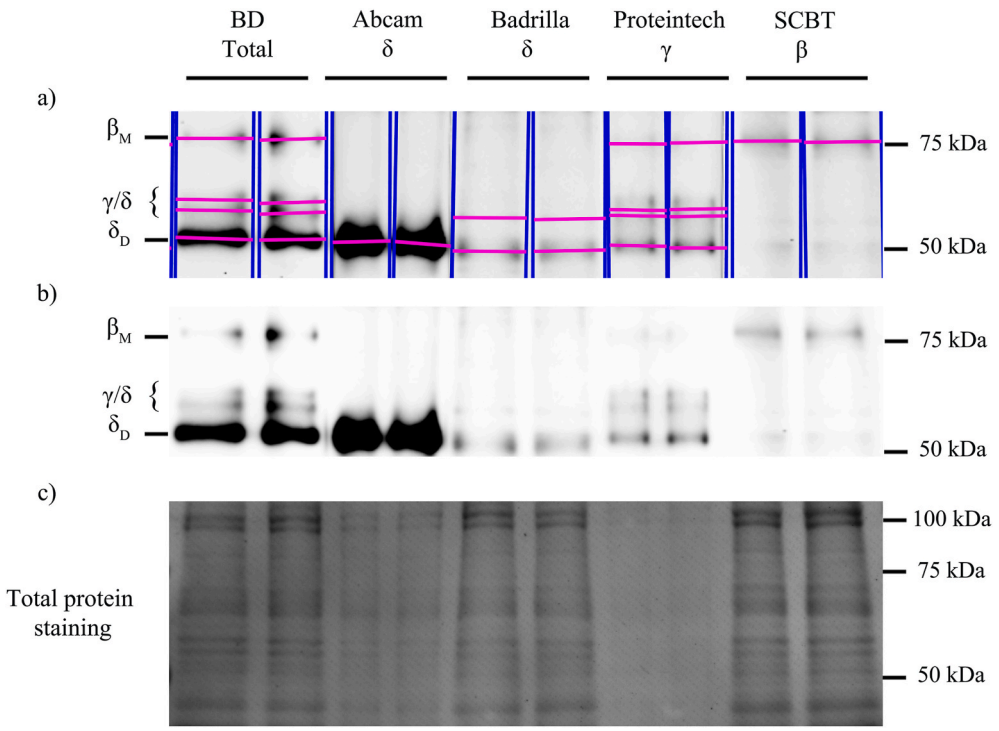

Fig. 2. CaMKII isoforms identification through immunoblotting in human skeletal muscle from a healthy participant obtained under resting conditions. **a) Representative image** obtained using Image Lab© software 6.0.1 (Bio-Rad) employing Lanes and Bands and MW Analysis Tools. The continuous purple line denotes the selected band used to calculate the molecular weight. Blue vertical lines delimit each lane. **b) Representative immunoblot** employing five commercial antibodies: BD Total, Abcam δ, Badrilla δ, Proteintech γ and SCBT β. **c) Total protein staining** using Reactive Brown 10. Isoforms (β_M, γ/δ and δ_D) are depicted on the left side of the panel. The band migration markers indicate the molecular weight in kDa.
BD Total: anti-CaMKII total (no. 611292, BD Biosciences); Abcam δ anti-CaMKII delta (no. 181052, Abcam); Badrilla δ anti-CaMKII delta (no. A010-55AP, Badrilla); Proteintech γ: anti-CaMKII Gamma (no. 12666-2-AP, Proteintech); SCBT β: anti-CaMKII Beta (no. 376828, Santa Cruz Biotechnology). Note that the antibody targeting the γ isoform displays high cross-reactivity with β and δ CaMKII, being unable to specifically resolve the γ isoform.

with the antibody for the total CaMKII. This was done as an additional assay to validate the bands obtained using the same PVDF membranes. After a 30-min incubation at room temperature (RT) in 2 % (v/v) sodium dodecyl sulfate containing 100 mM β-mercaptoethanol [46], the membranes were washed with TBS-T and stained with Reactive Brown 10 or Ponceau S for total protein presence confirmation. Following a 1-h blocking with 5 % skimmed milk, membranes were incubated with an HRP-conjugated anti-rabbit diluted 1:5,000–1:20,000 in 5 % skimmed milk blocking buffer for 1 h (no. 111-035-144, Jackson ImmunoResearch; SC2004, Santa Cruz Biotechnology). Afterwards, Clarity™ Western ECL Substrate (Bio-Rad) was added for subsequent visualisation (ChemiDoc™ Touch Imaging System, Bio-Rad), aiming to verify the effectiveness of antibody removal by assessing the absence of signal from the previously probed antibodies. Then, membranes were blocked again in 4 % BSA and reprobed for 12–15 h at 4 °C with anti-CaMKII total diluted 1:2,000 in 4 % BSA (no. 4436, Cell Signaling Technology). The HRP-conjugated anti-rabbit diluted 1:5,000 in 5 % skimmed milk (no. 111-035-144, Jackson ImmunoResearch) was incubated for 1 h, and the visualisation of the second protein was executed following the same procedural steps. See Tables S1 and S2, as well as the Appendix, for a detailed description of the materials and their composition.

3. Results

3.1. Identification and molecular weight determination of CaMKII isoforms from experiments 1, 2, 3 and 4

We examined the CaMKII isoform composition of human skeletal muscle by immunoblot analysis to determine the MW of each isoform using the 8 commercial antibodies (Figs. 1 and 2). Due to the inherent lack of exact measurements, the data shown has been rounded to the nearest unit separately for each isoform across experiments 1, 2, 3 and 4. Based on SDS–PAGE mobility [30,32–35,37], we identified the band with the lowest MW as δ_D (54.2 ± 2.1 kDa, n = 51). The group of intermediate bands above δ_D, exhibiting a slightly higher MW, was designated as γ/δ (59.0 ± 1.2 kDa, n = 45 and 61.6 ± 1.3 kDa, n = 45). The band with the highest MW corresponded to the β_M isoform (76.0 ± 1.8 kDa, n = 47).

3.2. Additional human experiments to verify CaMKII isoforms

In experiment 3, strength training induced a shift from MHC-IIx to MHC-IIa, driven by changes in sarcoplasmic [Ca^{2+}] sensed by CaMKII [14]. This intervention enabled the detection of changes in $pThr^{287}$-δ_D CaMKII, which is positively associated with muscle hypertrophy and the number of repetitions performed during training and negatively with the changes in MHC-IIx. In experiment 4, ROS produced during exercise until exhaustion triggered ROS-mediated signalling, leading to the upregulation of NRF2 and Thr^{287} CaMKII phosphorylation [3]. The close positive association between NRF2 total/Keap1 and $pThr^{287}$ CaMKII further validates this model for studying CaMKII isoforms.

3.2.1. Experiment 3: Identification of phosphorylated Thr^{287} CaMKII incubation, membrane stripping, and Total-CaMKII reprobing in human skeletal muscle

In the biopsies obtained before and after strength training (experiment 3, Fig. 3) [14], the membranes were first incubated with $pThr^{287}$-CaMKII (exposure time 110 s, binning 4 × 4) (Fig. 3a, upper panel). After a successful 30-min stripping (Fig. 3b, upper panel) of the PVDF membrane (exposure time 371 s, binning 4 × 4), the presence of

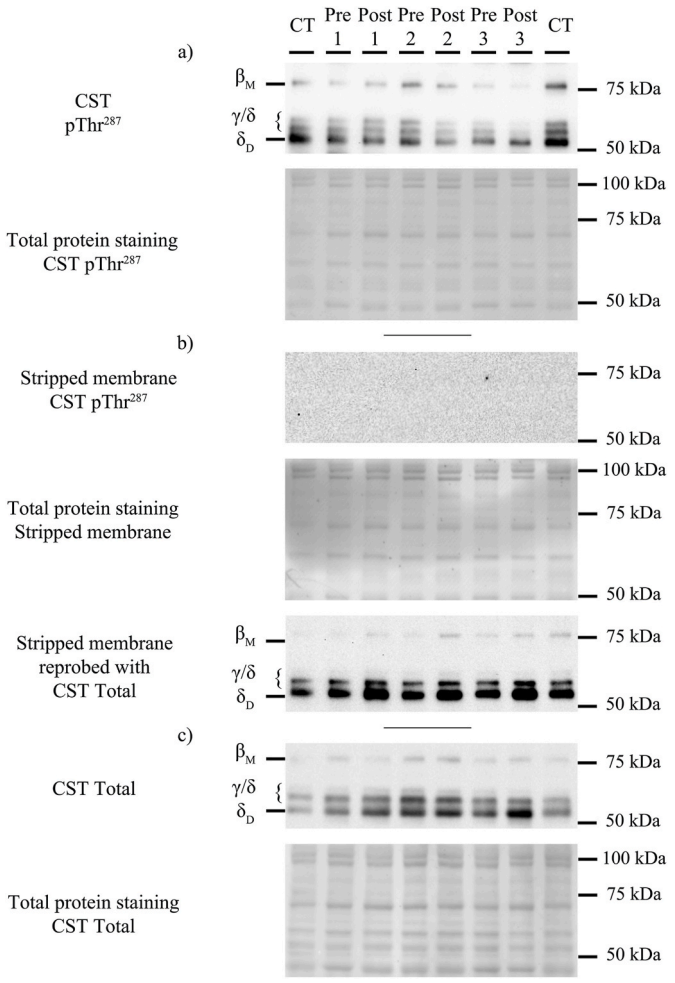

Fig. 3. Immunoblotting steps for experiment 3, including three representative young male participants assessed before and after a strength training programme. a) Membrane 1. Upper panel: Incubation with pThr^{287}-CaMKII (exposure time 110 s, binning 4 × 4); Lower panel: Total protein staining using Reactive Brown 10. **b) Stripped membrane 1**. Upper panel: Chemiluminescent signal (exposure time 371 s, binning 4 × 4) of pThr^{287}-CaMKII after 30 min of stripping with custom-made stripping buffer; middle panel: Total protein staining using Reactive Brown 10 after 30 min of stripping; lower panel: Reprobing with total-CaMKII (exposure time 720 s, binning 2 × 2) of the stripped membrane. **c) Membrane 2**. Upper panel: Incubation with total-CaMKII (exposure time 37 s, binning 4 × 4); lower panel: Total protein staining using Reactive Brown 10. Isoforms (β_M, γ/δ and δ_D) are depicted on the left side of the panel. The band migration markers indicate the molecular weight in kDa. CST pThr^{287}: anti-phospho-CaMKII-Thr^{287} (no. 12716, CST), CST Total: anti-CaMKII total (no. 4436, CST), CST: Cell Signaling Technology, CT: human control sample (non-experimental, Pre: biopsy sample taken before a strength-training programme, Post: biopsy sample taken after a strength-training programme.

proteins was confirmed by staining with Reactive Brown 10 (Fig. 3b, middle panel). Next, the membrane was reprobed with the total-CaMKII antibody for band validation (Fig. 3b, lower panel). The resulting bands from the pThr^{287}-CaMKII were positioned in the lower region, encompassing δ_D, followed by γ/δ bands, while the upper region featured the β_M isoform. This observed pattern was consistent when employing total-CaMKII in both the stripped membrane (exposure time 720 s, binning 2 × 2 (Fig. 3b, lower panel) and the non-stripped membrane (exposure time 37 s, binning 4 × 4) (Fig. 3c, middle panel).

3.2.2. Experiment 4: Identification of isoforms using an antibody specific for oxidised-Met$^{281/282}$ -CaMKII: incubation, membrane stripping, and Total-CaMKII reprobing in human skeletal muscle

For the acute exercise biopsies (experiment 4, Fig. 4) [3,4], membranes were initially subjected to incubation with an antibody directed against oxidised-Met$^{281/282}$-CaMKII (exposure time 72 s, binning 2 × 2) (Fig. 4a, upper panel). After a successful 30-min stripping (Fig. 4b, upper panel) of the PVDF membrane (exposure time 720 s, binning 4 × 4), the presence of proteins using Reactive Brown 10 was confirmed (Fig. 4b, middle panel). Next, the membrane was reprobed with the antibody against total-CaMKII for verification (Fig. 4b, lower panel). The band stained with anti-oxidised-Met$^{281/282}$-CaMKII with lower MW comprised δ_D, followed by γ/δ bands, while the β_M isoform laid at a higher MW. This distinctive pattern was consistently manifested when employing the antibody against total-CaMKII in both the non-stripped membrane (exposure time 37 s, binning 4 × 4) (Fig. 4b, lower panel) and the non-stripped membrane (exposure time 30s, binning 2 × 2) (Fig. 4c, upper panel).

4. Discussion

Most studies in human skeletal muscle have distinguished only two main CaMKII isoforms: γ/δ and β_M. γ/δ (γ and δ, analysed conjointly) represented the sum of the electrophoretic bands with MW ranging between 50 and 65 kDa, while β_M corresponded to a single upper band with an MW close to 75 kDa [34,37–39]. Notably, alternative characterisations have defined CaMKII as a singular 50 kDa band [47], a band range spanning 50–60 kDa [40] omitting β_M isoform, or a band assembly without specifying MWs [5,41,42,48]. In this context, we present an easy and cost-effective approach to distinguish different CaMKII β_M, γ/δ and δ_D isoforms, suitable for implementation in most laboratories. The immunoblotting assessment of phosphorylated and oxidised CaMKII with specific antibodies, followed by stripping and subsequent positive incubation of the specific band using an antibody that binds with all CaMKII, verified the band identification. Despite the technical limitations of immunoblotting, the usage of 8 different commercial antibodies conferred robustness to the assays. The parallel findings in the banding pattern across membranes and the reproducibility of findings across experiments and conditions underscore the overall validity of the experimental procedures.

The β_M CaMKII is abundantly expressed in mouse skeletal muscle [19,22], which Tombes et al. [19] identified as a 72.7 kDa band, i.e., slightly less than the 76.0 ± 1.8 kDa observed in our experiments. However, our results align with the studies in human skeletal muscle, which consistently report β_M CaMKII band above the molecular marker of 70 kDa [3,4,14,34,35,37–39]. Studying β_M in human skeletal muscle is of significant interest, as it forms a complex with the glycolytic machinery at the sarcoplasmic reticulum, regulating glycolytic metabolism, calcium transport, and muscle excitation-relaxation [6].

The δ_D CaMKII isoform is exclusive of skeletal muscle, theoretically weighing 58.4 kDa [19], while our data indicate that the corresponding MW for human skeletal muscle is 54.2 ± 2.1 kDa. We tested two different δ-specific CaMKII antibodies. Our assays indicated that the δ CaMKII Badrilla and δ CaMKII Abcam identified a band in 50.9 ± 0.31 kDa and 51.7 ± 0.08 kDa, respectively. In humans, the basal protein expression level of the δ_D isoform increases following a strength training program in parallel with the degree of muscle hypertrophy elicited by the training program [14]. Likewise, the specific δ CaMKII expressed in rodent myocardiocytes, the δ_B, plays an essential role in hypertrophy after myocardial ischemia/reperfusion injury [49,50]. Our human studies support the emerging role of the δ_D CaMKII isoform as the most exercise-responsive CaMKII isoform in skeletal muscle [3,4], which appears to contribute to the NF-κB [4] and NRF2/Keap1 [3] signalling activation.

The γ CaMKII isoform displays widespread expression with diverse spliced variants across neural and nonneural tissues [26,28,30,51]. γ_B

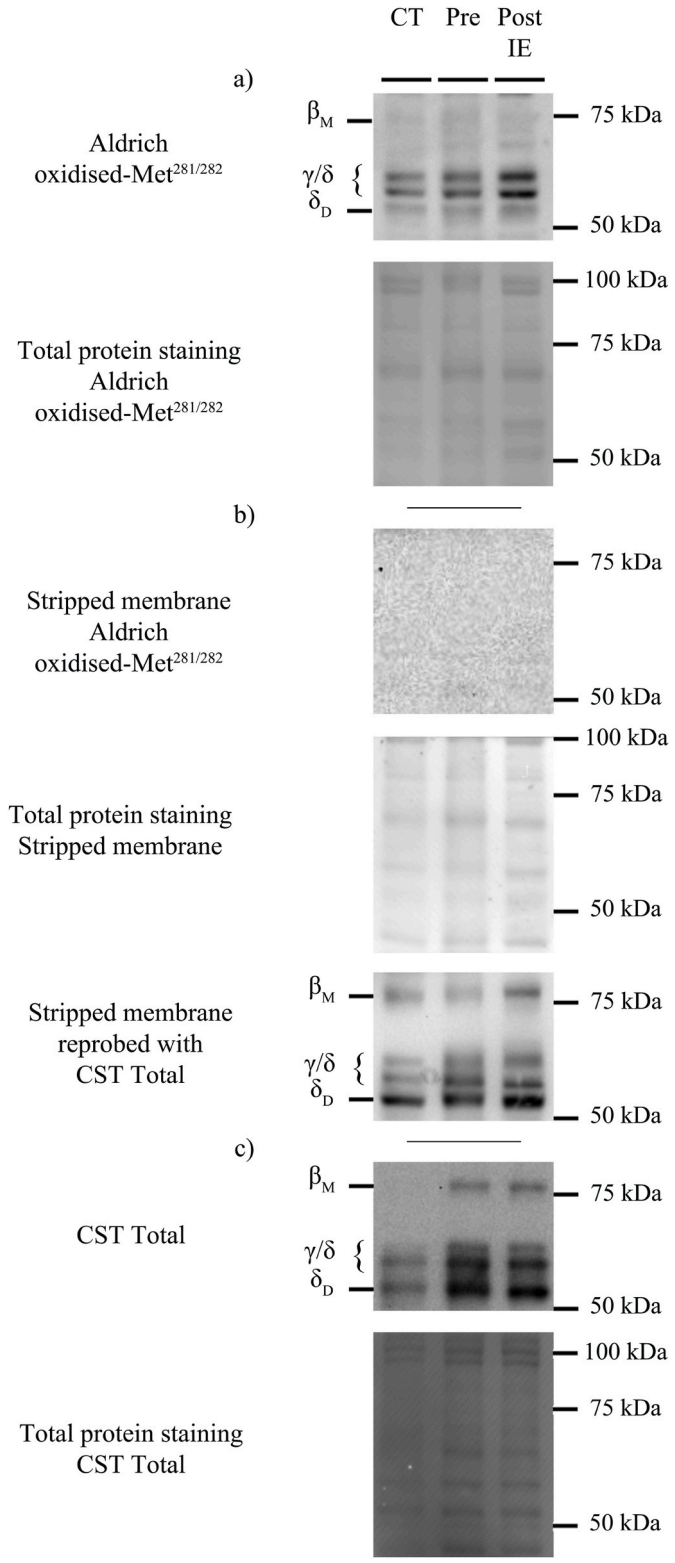

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Fig. 4. Immunoblotting steps for experiment 4, including one representative young male participant who underwent an incremental exercise until exhaustion. a) Membrane 1. Upper panel: Incubation with oxidised-Met$^{281/282}$-CaMKII (exposure time 72 s, binning 2 × 2); Lower panel: Total protein staining using Reactive Brown 10. **b) Stripped membrane 1**. Upper panel: Chemiluminescent signal (exposure time 720 s, binning 4 × 4) of oxidised-Met$^{281/282}$-CaMKII after 30 min of stripping with custom-made stripping buffer; middle panel: Total protein staining using Reactive Brown 10 after 30 min of stripping; lower panel: Reprobing with total-CaMKII (exposure time 139 s, binning 2 × 2) of the stripped membrane. **c) Membrane 2**. Upper panel: Incubation with total-CaMKII (exposure time 30 s, binning 2 × 2); lower panel: Total protein staining using Ponceau S. Isoforms (β_M, γ/δ and δ_D) are depicted on the left side of the panel. The band migration markers indicate the molecular weight in kDa
Aldrich oxidised-Met$^{281/282}$: anti-oxidised-Met$^{281/282}$-CaMKII (no. 07–1387, Sigma Aldrich), CST Total: anti-CaMKII total (no. 4436, CST), CST: Cell Signaling Technology, CT: human control sample (non-experimental, Pre: biopsy sample taken at rest, Post IE: biopsy sample taken after incremental exercise until exhaustion.

CAMKII protein [30] and γ_C mRNA have been reported in the skeletal muscle of rats [26,28] and different human tissues [51]. As Tombes et al. [19] outlined, the γ_C variant is ubiquitously present in tissues with a 56 kDa MW. The γ_B is present in T-cells, smooth muscle, astrocytes and islets with a 58.4 kDa MW. The δ_A CaMKII isoform has been reported as a band positioned below β_M CaMKII in rodent skeletal muscle [36] being involved in the modulation of muscle fibre type-specific gene expression, primarily via the phosphorylation of histone deacetylase 4 (HDAC4), enabling the transition from a fast-to-slow muscle phenotype [52]. The MW for this band has been postulated to lay close to 60 kDa [19]. In the present investigation, we identified γ/δ as the two upper bands below β_M CaMKII, with MWs of 59.0 ± 1.2 kDa and 61.6 ± 1.3 kDa, respectively. The γ CaMKII antibody used here was not isoform-specific due to its cross-reactivity with all the CaMKII isoforms, even if a minimal amount of protein was loaded (0.5 μg of human skeletal muscle lysate). The δ CaMKII purchased from Badrilla targeted a band at 57.9 ± 0.42 kDa (n = 4), which was not detected by δ CaMKII purchased from Abcam. Discerning between δ_A, γ_C, and γ_B splicing variants was not achievable with Western blot, despite using antibodies that claimed specificity. Further research is needed to distinguish between these CaMKII isoforms, targeted by total and phosphorylated CaMKII antibodies.

4.1. *Specificity of CaMKII isoform antibodies*

High homology between isoforms results in cross-reactivity with isoform-specific antibodies. Most studies using the four specific isoform antibodies report bands without specifying the MW at which these bands were observed.

The β CaMKII antibody has been tested in pig skeletal muscle without reporting MW [53]. In Fan et al. [54], β CaMKII is reported at 57 kDa in PC12 cells (neural tissue). Our study revealed that the specific antibody against β CaMKII marked a single band, which precisely aligned at MW corresponding to a similar band marked by the antibodies directed against the total, phospho- and oxidised CaMKII. The MW (76.0 ± 1.8 kDa) concured with that reported for β_M CaMKII in human skeletal muscle by Christiansen et al. [38] and Thomassen et al. [39] using pThr^{287}-CaMKII (no. 12716, Cell Signaling Technology) and total-CaMKII (no. 4436, Cell Signaling Technology) and total-CaMKII (no. 611293, same as 611292, BD Biosciences) and pThr^{287}-CaMKII (no. 3361, Cell Signaling Technology, discontinued).

In rodents, the δ_D CaMKII has been described in skeletal muscle [19] and cardiac myocytes [49,50]. Previous evidence using the antibody purchased from Abcam reports a 56 kDa band in HL-1 myocytes [55], while non-specific MW is reported in other studies [56,57]. In the present investigation, δ CaMKII from Abcam only detected a clear single band at 51.7 ± 0.08 kDa, which concurred with the Total and pThr^{287} CaMKII antibodies at this MW. The δ CaMKII from Badrilla detects a group of bands in the atrial tissue of rats [58] without specifying the MW. Our study detected two bands, the lowest at 50.9 ± 0.3 kDa, which coincided with the Abcam δ CaMKII band, and the highest at 57.9 ± 0.42 kDa. Although no MW is defined, the γ CaMKII antibody has been knock-down validated in multiple myeloma cells [59]. Other studies

report a single band at 59 kDa [60,61] or without MW [62]. The γ CaMKII antibody has also been used to target total CaMKII [63–65]. Even when loading a low amount of protein (0.5 μg of human skeletal muscle in our study), the γ CaMKII antibody had high cross-reactivity with β and δ CaMKII. Despite the characterisation of β_M, γ_D-δ_D and δ_A [36] and β_M, γ_B, δ_A and δ_B [30] in rat skeletal muscle, discerning between γ and the upper δ CaMKII band was not achievable using current commercial antibodies. Therefore, γ/δ have been analysed conjointly in the present investigation.

4.2. Limitations

This study has several limitations. Although none of the bands was immunoprecipitated and submitted to mass spectrometry, most commercial antibodies have been shown to be specific and have been used in numerous previous studies. Besides, the experiments executed in the present investigation took advantage of protein modifications (phosphorylation and oxidation) that are specific for CaMKII and can be tracked with the antibodies used. Greater knowledge could be gained regarding the regulation of the total protein changes by adding measurements of gene expression. However, the protein levels may change independently from changes in mRNA expression due to changes in translation efficiency or protein degradation rate, as reported for Keap1 and Nrf2 [66]. Additional information regarding gene expression can be obtained using the MetaMex tool, which allows the generation of meta-analysis of multiple experiments using published transcriptomic data from skeletal muscle biopsies obtained in human experiments [67]. In response to acute and chronic exercise, no significant changes have been observed in mRNA levels of CAMK2B (beta isoform) or CAMK2G (gamma isoform) [67]. In agreement with our previous publication [14], an increased CAMK2D (delta isoform) mRNA expression has been observed for resting muscle biopsies obtained 72 h after the end of strength training programmes lasting between 12 and 26 weeks, compared to the values observed before training [67].

In summary, this study shows a practical immunoblotting approach for discerning the CaMKII isoforms β_M, γ/δ and δ_D in human skeletal muscle with commercial antibodies. Remarkably, our findings confirm the MW of β_M CaMKII and allows distinguishing between γ/δ and δ_D CaMKII, the latest being the most responsive isoform to ROS stimulation. It has been confirmed that, as indicated by the manufacturer, the γ CaMKII antibody displays cross-reactivity with all CaMKII isoforms, not allowing distinguishing between γ/δ isoforms. Correctly identifying CaMKII isoforms is crucial for elucidating the functional role of each isoform. Here we have demonstrated that exercise can be used to elicit specific posttranslational modifications, such as phosphorylations and oxidations, which allow the identification of specific bands by multi-targeting them with different antibodies after stripping and reprobing.

Disclosure summary

The authors have nothing to disclose.

Data availability statement

The data that support the findings of this study are available on request from the corresponding authors.

CRediT authorship contribution statement

Miriam Martinez-Canton: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation. **Angel Gallego-Selles:** Writing – review & editing, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Victor Galvan-Alvarez:** Writing – review & editing, Methodology, Investigation, Data curation, Conceptualization. **Eduardo Garcia-Gonzalez:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Giovanni Garcia-Perez:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Alfredo Santana:** Writing – review & editing, Methodology, Investigation, Conceptualization. **Marcos Martin-Rincon:** Writing – review & editing, Supervision, Software, Resources, Methodology, Formal analysis, Data curation, Conceptualization. **Jose A.L. Calbet:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary materials and methods

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