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Two splice variants of CaMKII-anchoring protein are present in the sarcoplasmic reticulum of rabbit fast-twitch muscle

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Abstract

Anchoring protein αKAP targets calmodulin kinase II (CaMKII) to the sarcoplasmic reticulum (SR), and in the rabbit is a substrate of CaMKII itself in fast-twitch, but not in slow-twitch muscle. This work was aimed at elucidating the molecular basis for differential phosphorylation of αKAP. Here we show that two, immunologically related, size forms (23 and 21 kDa) of αKAP are present in fast-twitch muscle SR in a 3:1 stoichiometry. Phosphorylation experiments identified the shorter form as the CaMKII specific substrate. Both forms are shown to be stably integrated into the holoenzyme. Two splice variants of αKAP were found in rabbit fast-twitch muscle and only one in slow-twitch muscle, using RT-PCR. Mobilities on SDS–PAGE are those expected. The shorter splice variants lacks the 33-nucleotide sequence inserted by alternative splicing present in full-length αKAP, akin to differences between variants A and B of brain αCaMKII. The absence of the 11-amino acid sequence creates a novel CaMKII phosphorylation site. Taken together our results show that alternative splicing regulates αKAP phosphorylation in a fiber-type specific manner.
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Keywords: Skeletal muscle; Sarcoplasmic reticulum; CaM kinase II; Anchoring protein; αKAP splice variants

Ca^{2+}/calmodulin (CaM)-dependent protein kinase II (CaMKII) is a multifunctional Ser/Thr kinase, with substrates found in nuclei, cytoskeletal, and membrane compartments [1], including the sarcoplasmic reticulum (SR) of mammalian twitch fibers [2,3]. CaMKII is encoded by four genes (α, β, γ, and δ) that also exhibit alternative splice variants. The γ and δ subunits are expressed in most tissues, whereas the α isoforms are most prominent in neural tissues [4,5]. In skeletal muscle in which α subunits are not expressed, CaMKII is composed primarily of γ and δ isoforms (60 kDa) [6–8], and a varying proportion of a 72 kDa isoform of the β subunit (β_M) [6], according to the animal species and the type of muscle [9,7].

In mammalian slow-twitch muscles CaMKII associated to longitudinal SR plays a key role in the regulation of Ca^{2+}-transport through phosphorylation of (slow-twitch/cardiac) SERCA2a isoform, as well as of regulatory protein phospholamban, albeit with the exception of small rodent species [10]. A role for CaMKII in the regulation of SR Ca^{2+}-release in rabbit fast-twitch muscle is directly supported by the evidence that CaMKII acts as a negative modulator of native RyR1/Ca^{2+}-release channel [11], an effect which, however, may be mediated by the phosphorylation, not of RyR1 itself, but rather of triadin [8,12].

Junctional triads in skeletal muscle fibers may be viewed as interior synapses, involving electromechanical coupling between the α1 subunit of dihydropyridine receptor, localized on the transverse tubules (TT), acting mainly as a voltage sensor, and RyR1/Ca^{2+}-release channel placed on the junctional membrane portion of terminal cisternae (TC) [13]. The association of CaMKII to TC would allow it to respond to brief Ca^{2+} transients, and to act as a mediator of activity-induced muscle plasticity, such as, for example, post-tetanic potentiation

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of isometric twitch force, as found in fast-twitch, muscles [13,14].

In recent years, CaMKII-anchoring proteins [αKAP, F-actin, and densin-180, see 15,16] have been identified, serving to target it to specific subcellular sites. Targeting of CaMKII to the SR of mammalian skeletal muscle was shown to involve αKAP, a truncated product of αCaMKII gene transcribed by a different promoter within the αCaMKII gene, and the first specific CaMKII anchoring protein to be identified [17].

This study originated from the observation that, in the rabbit, αKAP acted as a specific substrate of CaMKII itself in fast-twitch, but not in slow-twitch muscle [7]. We have used a wide variety of experimental methods, from SDS–PAGE, Western blot-analysis, co-immunoprecipitation, and chromatographic methods to RT-PCR analysis, to elucidate the molecular basis for the differential phosphorylation of αKAP. Here we show that αKAP is expressed in rabbit fast-twitch muscle in two size forms that are generated by alternative splicing, and differ only in the 11-amino acid insert between the linker region with N-terminal domain and the association domain [17]. Furthermore, we show that CaMKII-mediated phosphorylation in native SR membranes of rabbit fast-twitch muscle is restricted to the αKAP isoform lacking the insert, which was unforeseeable at the time of our own initial findings [7].

Materials and methods

All chemicals were of analytical grade. Molecular mass standards were purchased from BDH Lab. (Poole, England). [γ-^{32}P]ATP (3000–6000 Ci/mmol) and [^{3}H]ryanodine (77–87 Ci/mmol) were from NeN (Du Pont De Nemours, Germany). Hog brain calmodulin was purchased from Boehringer (Mannheim, Germany). CaMKII inhibitor KN-93 was purchased from Calbiochem (San Diego, CA, USA). Potato acid phosphatase (10 U/mg pr) was from Sigma–Aldrich (St. Louis, MO, USA).

Tissue harvesting. Experimental animals (New Zealand male adult rabbits) were lawfully acquired, and taken care of, in the Animal Care Facility of the Department of Experimental Biomedical Sciences of the University of Padova, in compliance with Italian Law (D.L. September 27, 1992, No. 116), and circular letter of the Ministry of Public Health, (April 22, 1994 No. 8). Animals were adequately anesthetized and then killed by cervical dislocation. Rabbit adductor and soleus were used as representative fast-twitch and slow-twitch muscle, respectively [7,9,18]. The additional (mixed) muscles used were rabbit diaphragm and human vastus lateralis muscle. The latter was obtained from the Department of Oncological and Surgical Sciences, School of Medicine of the University of Padova on application to the same Department. The tissue had been removed from an adult male, 67-year-old patient, in the course of surgery of a rhabdomyosarcoma in the thigh, with the freely given, informed consent of the patient. The sample was collected in liquid nitrogen and store-frozen before use at the Department of Experimental Biomedical Sciences.

Isolation and purification of the SR and of junctional face membrane. SR membranes were isolated from muscle homogenates, by the method of Saito et al. [19] with slight modifications [20], and fractionated by isopycnic sucrose-density centrifugation to yield four distinct fractions, labeled R1–R4 from the top to the bottom of the gradient. Isolated TC vesicles were depleted of endogenously bound CaM by incubating for 30 min on ice in a medium buffered at pH 7.4 with 5 mM imidazole and containing 0.3 M sucrose, 0.2 mM EGTA, and 0.006 mM $CaCl_2$ (final pCa: 9), and by centrifuging at 150,000*g* 90 min [21]. Junctional face membrane (JFM) was obtained by incubating sucrose-density purified TC in a medium having the same basal composition, to which Chaps was added to a final concentration of 0.25% Chaps (0.7–1 detergent to protein ratio) [21]. The Chaps-insoluble membrane fraction was further subfractionated on sucrose-step gradient and using experimental conditions identical to those used to fractionate SR membranes [9,22]. Purified JFM was collected as a pellet at the bottom of the tube. Membrane fractions resuspended in buffered 0.3 M sucrose were stored at −80 °C, until use. Protein concentration was determined by the Folin reaction [23], using bovine serum albumin as a standard.

Phosphorylation. TC were phosphorylated by endogenous CaMKII with 400 μM [γ-^{32}P]ATP (specific radioactivity 0.10 Ci/mmol), using experimental conditions identical to those reported previously [24]. The concentration of free Ca^{2+} was kept constant (pCa 4). Unless stated otherwise (see legends to figures), the concentration of CaM was 1 μM. Incubation was at 0–4 °C for various times, as reported in the legends to Figures. Adding SDS-solubilizing buffer to samples quenched the reaction. SDS-gels after being dried were analyzed by autoradiography (16 h exposure), by a Model GS-250 Molecular Imager (Bio-Rad) [8,12], or by a Packard Instantimager for quantification of ^{32}P-incorporation into protein [7,24].

Protein dephosphorylation. Isolated TC were phosphorylated for 10 min with [^{32}P]ATP in standard assay medium, containing 1 μM CaM. As reported in detail in the legends to Fig. 5, protein dephosphorylation by potato acid phosphatase was carried out in the same medium at 30 °C.

Purification of CaMKII by CaM–Sepharose affinity-chromatography. CaMKII was co-solubilized with SR Ca^{2+}-pump membrane by partial extraction of sucrose-density purified TC with 0.25% Chaps [21] and purified by CaM–Sepharose affinity chromatography, as described [7].

Immunoprecipitation. Immunoprecipitation experiments were carried out, as described by Brocke et al. [25]. Hundred μl aliquots of affinity-purified CaMKII were dissolved in 0.5 ml of 50 mM Tris/HCl, pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% Na-deoxycholate, and 0.1% SDS, and then incubated for 2 h in cold room on a roller with goat polyclonal anti-δ CaMKII I-16 antibody (1:1000 dilution). Protein G-Agarose (40 μl) was added to the mixture incubated for 1 h at 0–4 °C and sedimented for 5 min using an Eppendorf centrifuge. After three washes with cold PBS/0.25% NP-40, pellets were solubilized with 100 μl of Laemmli's SDS-buffer for 5 min in boiling water. Blots were developed using enhanced chemiluminescence (ECL, Amersham). Staining of protein-G on blots is due to the binding interaction between this protein and primary anti-δ CaMKII antibody raised in the goat, to which secondary, HRP-conjugated, rabbit anti-goat antibody will bind.

Gel electrophoresis and immunoblotting. SDS–PAGE was carried out according to Laemmli [26], using a Bio-Rad Protean apparatus, with 16-cm-long slab gels. Apparent Mrs were calculated from a graph of relative mobilities versus log Mr of standard proteins. Blots of proteins transferred onto nitrocellulose were probed [24] with the following antibodies:

(1) *Mouse monoclonal antibodies*. (a) Triadin (ABR, Golden, CO, USA); (b) RyR1 (ABR); and (c) A-1 antibody (Santa Cruz Biotech. CA, USA), raised against aminoacids 303–478 of αCaMKII of mouse origin. It was verified that A-1 antibody did not cross-react with β, γ, or δ CaMKII.

(2) *Rabbit polyclonal antibodies.* (a) M-176 antibody(Santa Cruz Biotech.). This antibody was raised against epitopes corresponding to aminoacids 303–478 mapping at the C-terminus of mouse CaMKII. Using isolated SR membranes and Western blot techniques, it was ascertained that this antibody was cross-reactive with γ and δ CaM-

KII. (b) Anti-ACTIVE CaMKII pAb (Promega Corporation, Madison, WI, USA). This antibody was raised against the phosphothreonine 286 (pT^{286})-containing peptide and reacts selectively with CaMKII phosphorylated at Thr-286/287.

(3) *Goat polyclonal antibodies.* (a) Anti-δ and anti-γ CaMKII (Santa Cruz Biotech.). It is well established that anti-γ CaMKII cross-reacts with β_M CaMKII [9,7]; (b) I-16 antibody (Santa Cruz Biotech.), recognizing an epitope mapping near the carboxyl terminus of αCaMKII of mouse origin. Based upon sequence homology studies between α CaMKII and αKAP [27], tied up with the information obtained from the manufacturer in response to a specific inquiry on this antibody, the epitope recognized by this antibody was identified as part of the sequence: *ESACIAIRITQYLDAGGIPRTA*, corresponding to aa 147-168 of αKAP sequence [17,28]. This antibody does not cross-react with β, γ, or δ CaMKII. Antibody binding was detected by immunoenzymic staining [24]. Densitometry of immunostained protein bands was carried out, using a Bio-Rad GS-670 densitometer.

Identification of αKAP transcripts. We searched the GenBank database (http://www.ncbi.nlm.nih.gov/blast) for sequences homologous to the 25-amino acid, N-terminal hydrophobic sequence unique to αKAP, using the tBLASTtn algorythm. Sequences were translated using the DNA Strider 1.2 software (CEA, Gif-Sur-Yvette, Cedex, France).

Isolation of αKAP cDNA fragments. Total cellular RNA was extracted from rabbit adductor magnus and soleus muscles, using the Ambion RNA Wiz Kit, according to manufacturer's protocol. cDNA was synthesized using the Superscript First Strand System (Invitrogen, Carlsbad, CA) and oligo(dT) primers. PCR primers were designed based on the mouse αKAP sequence [17] (see Table 1). Nomenclature follows the published guidelines for mammalian genes [29]. cDNA was amplified using a nested PCR protocol, with the PCR core KIT (Roche, Basel, Switzerland). For the first reaction we used primers 1F and 284R, PCR conditions were 94 °C for 3 min, then 35 cycles of 94 °C for 30 s, 54 °C for 30 s, 72 °C for 30 s, and a final extension step at 72 °C for 7 min. 0.5% of the PCR product was then amplified using primers 34F and 251R with the same conditions as those for the first step except that the annealing temperature was set at 55 °C. Fragments were separated on a 12.5% polyacrylamide gel in TBE and visualized by staining with silver, as described [30]. Bands were also separated on a 3% agarose gel and excised, and DNA was recovered with a Qiaquick Gel Extraction Kit (Qiagen, Valencia, CA). The isolated PCR fragments were directly sequenced using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit and the 310 Automatic Sequencer (Applied Biosystem, Perkin–Elmer, Foster City, CA).

Table 1
PCR primer sequences

NAME	Nucleotides	Sequence
1F	1–20	ATGCTGCTCTTTCTCACGC
34F	34–53	CCTTGCCTGGTGTTGCTAAC
251R	251–242	CAGCTGATCGAAGCCATAAG
284R	284–265	GAGTCCTACACGAAGATGTG

Nucleotide 1 corresponds to the beginning of the coding region of αKAP cDNA.

Results

Evidence that αKAP is a specific substrate of CaMKII associated to the SR of rabbit fast-twitch muscle

We previously reported Ca^{2+}/CaM-dependent phosphorylation of αKAP in rabbit fast-twitch muscle SR [7]. That was definitively assessed, using CaM-depleted, sucrose density-purified TC vesicles (see Materials and methods) and by carrying the incubation at a fixed concentration of free Ca^{2+} (pCa 4), in the absence and in the presence of increasing concentrations of CaM

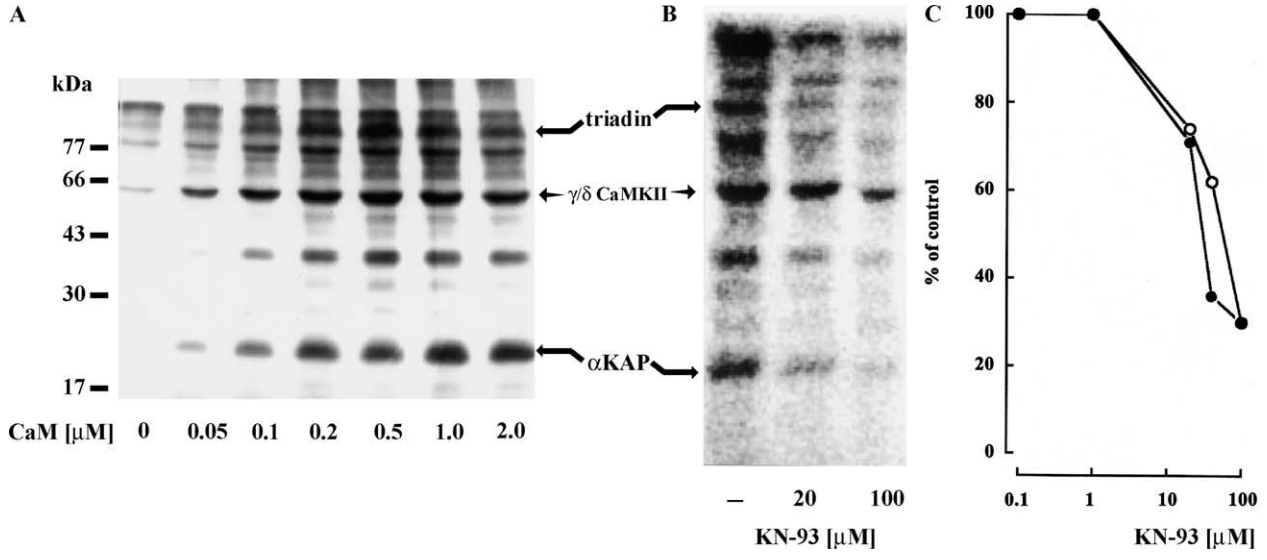

Fig. 1. Effect of CaMKII activation or inhibition on the pattern of protein phosphorylation in CaM-depleted TC vesicles from rabbit fast-twitch muscle (A and B). Autoradiographs of TC incubated with 400 μM [γ-^{32}P]ATP, in the absence or presence of 0.05–2 μM CaM (A), or in the presence of 0.2 μM CaM and KN-93 at the indicated concentrations (B). The expected mobilities of triadin, CaMKII subunits, and αKAP are indicated. (C) Dose-dependent inhibition by KN-93, at 1 μM CaM, of ^{32}P-incorporation into 60 kDa protein band comprised of γ/δ CaMKII (○) and into 21 kDa αKAP (●). Incubation was at 0–4 °C for 10 min. Proteins were resolved by 10–15% SDS–PAGE. Protein loading was 50 μg/lane (A) or 35 μg/lane (B and C). Quantitative determination of radioactivity (C) was done by computer-assisted analysis of each signal in dried gels (see Materials and methods).

(Fig. 1A). We found that phosphorylation of αKAP required the addition of CaM and was similar in CaM-dependency to CaMKII autophosphorylation and CaMKII-mediated phosphorylation of triadin [12,21]. Phosphorylation events were found to be inhibited by CaMKII inhibitor KN-93 [31]. In agreement with previous evidence that KN-93 inhibits CaMKII in a competitive fashion with CaM, the results in Figs. 1B and C show that inhibition of CaMKII autophosphorylation and activity by KN-93 was much higher in the presence of 0.2 μM CaM than at 1 μM CaM.

It is well demonstrated and agreed that CaMKII is maintained in an inactive state by an autoinhibitory regulatory domain, and that binding of Ca^{2+}/CaM relieves the autoinhibition, to the effect of increasing both kinase autophosphorylation and the phosphorylation of exogenous substrates. Autophosphorylation of Thr-286 at the α-subunit regulatory domain (or at Thr-287 for the other isoforms) occurs within the oligomeric complex, through the interaction between adjacent subunits that have bound Ca^{2+}/CaM, and is regarded to be uniquely responsible for the generation of an autonomous, Ca^{2+}-independent enzyme [4,1,32].

Fig. 2 describes the time-course of ^{32}P autophosphorylation of CaMKII associated to TC, at both 60 kDa γ/δ (Fig. 2A) and 72 kDa β_M subunits (Fig. 2C), based upon identification by specific Western blotting (see below, Fig. 3).

For assessing that phosphorylation of γ/δ subunits was at the "*autonomy*" site, we used Western blot techniques and a polyclonal, phospho-Thr-286/287-specific antibody. Transition of CaMKII associated to TC from its basal dephosphorylated state to phosphorylation state at Thr-287 (inset to Fig. 2B) appears to be very rapid and to be almost complete at 30 s. There is likewise evidence to suggest that Thr-287 stayed highly phosphorylated between 2 min and 10 min. What is most apparent is a slowing of the phosphorylation rate of αKAP after 2 min (Figs. 2A and B). Thus, ^{32}P phosphorylation of αKAP continued to rise, when phos-

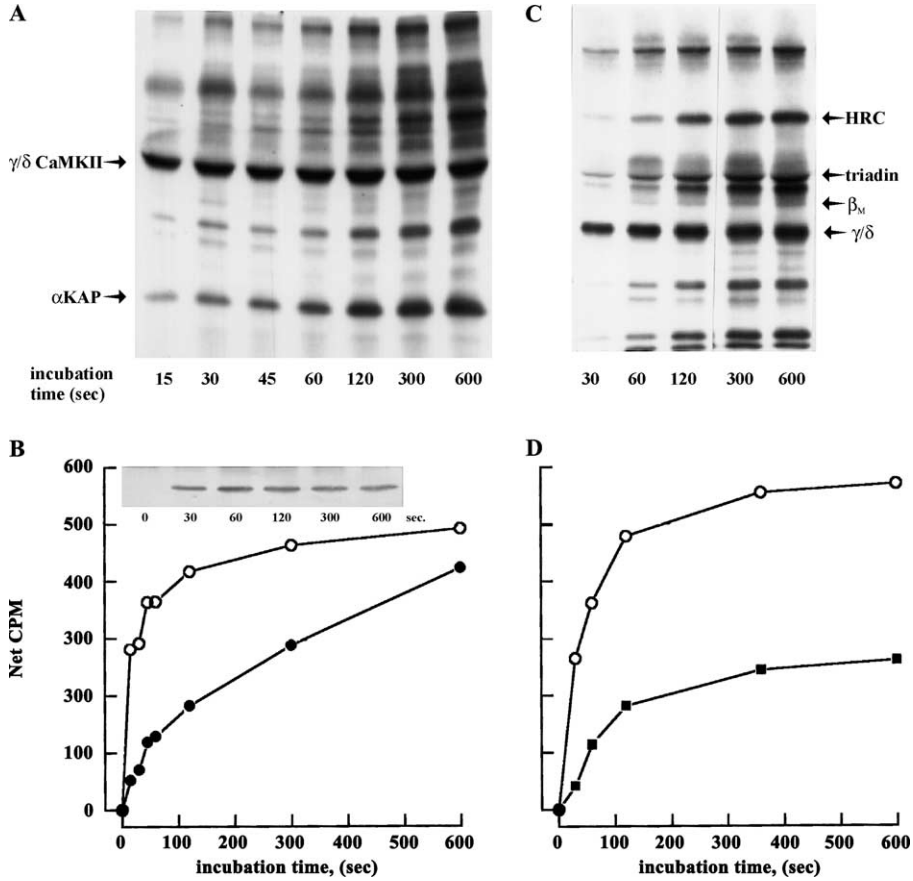

Fig. 2. Time-course of the autophosphorylation of CaMKII full-length subunits and of CaMKII-mediated phosphorylation of triadin and αKAP. TC vesicles from rabbit fast-twitch muscle were incubated in standard phosphorylation medium containing 1 μM CaM, for various times. Protein was resolved by 10–15% (A and B) or 5–10% SDS–PAGE (C and D). Protein loading was 50 μg/lane. ^{32}P-labeling of proteins was quantified (B and D), as in Fig. 1, (C) Symbols: (○) CaMKII; (●) αKAP; and (■) triadin. Inset to (B) Western blot analysis of γ/δ CaMKII, using specific anti-$pT^{286/287}$ antibody (see Materials and methods), of unincubated TC and of TC that had been incubated with 400 μM cold ATP in standard phosphorylation medium for the times indicated. SDS–PAGE (10–15%) was carried out in the presence of 1 mM $CaCl_2$, to the intent of improving the resolution of 60 kDa γ/δ CaMKII from 63 kDa calsequestrin, which undergoes an acceleration in mobility in the presence of $CaCl_2$.

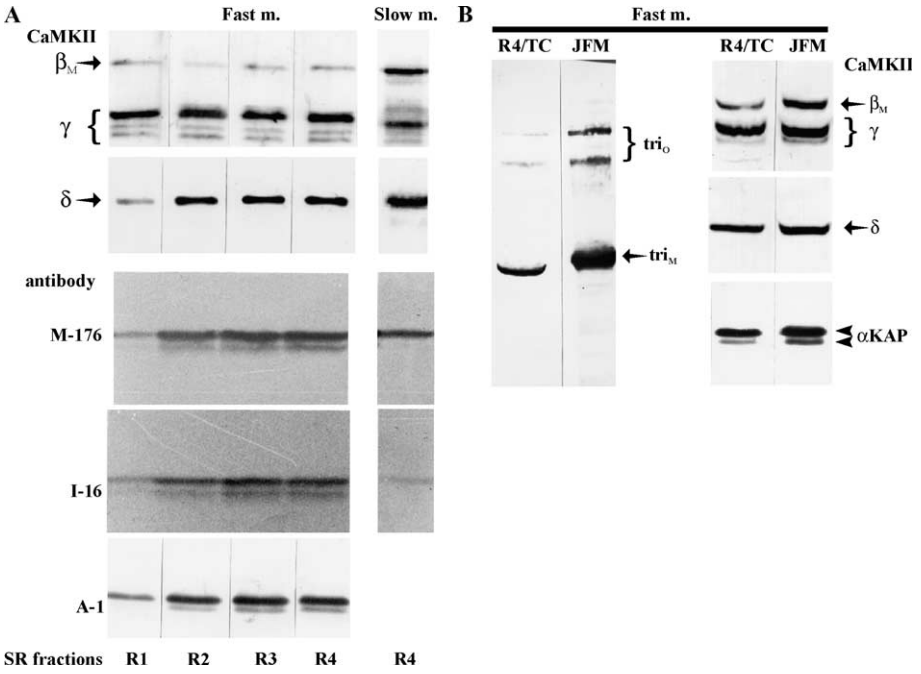

Fig. 3. Western blot analysis of the distribution of αKAP and CaMKII full-length subunits in isolated SR subfractions from rabbit fast-twitch and slow-twitch muscles. (A) Samples (30 μg/lane) of sucrose-density purified SR fractions (R1–R4) from rabbit fast-twitch muscle and of isolated TC (R4) from slow-twitch muscle were analyzed by 5–10% (CaMKII subunits) or by 10–15% (αKAP) SDS–PAGE. Blots were immunostained with the indicated antibody. CaMKII δ subunit was identified using subunit-specific antibody, whereas the γ and β_M CaMKII subunits were identified simultaneously, using a polyclonal antibody to γ CaMKII, previously shown to be cross-reactive with β_M (see Materials and methods). (B) Samples (50 μg/lane) of Chaps-purified JFM and of CaM-depleted TC vesicles (see Materials and methods), after 5–10% SDS–PAGE, were analyzed by immunoblotting with antibody to triadin. The position of triadin monomers (tri_M) and oligomers (tri_O) is indicated. Additional blots, after 10–15% SDS–PAGE, were probed with antibodies to γ CaMKII (cross-reactive with β_M), to δ CaMKII, and with M-176 antibodies to detect αKAP peptides.

phorylation of triadin had attained almost maximal levels (Figs. 2C and D).

Evidence that two size forms of αKAP are present in fast-twitch muscle SR and that CaMKII selectively phosphorylates the small size form

Western blot analysis of αKAP in isolated, sucrose-density purified SR fractions was carried out in combination with a highly resolving SDS-gel system, as it was in autoradiographic studies described in the Introduction. As shown in Fig. 3A, the results were the same, using two kinds of polyclonal antibodies (M-176 and I-16), or a monoclonal antibody (A-1), raised against epitopes localized to the association domain of mouse αCaMKII. All antibodies specifically recognized a 23–21 kDa peptide doublet in SR-derived fractions from the sucrose-step gradient of muscle homogenates (R2 to R4), as well as in CaM-depleted TC and Chaps-purified JFM from rabbit fast-twitch muscle (Fig. 3B), whereas a single peptide was detected at 23 kDa in isolated TC from rabbit soleus muscle (Fig. 3A) (identical results were obtained with fractions R2–R3 of predominant longitudinal SR membrane origin, not shown). Neither peptide was immunodetected in post-microsomal supernatants (not shown).

The ratio between the 23 kDa band and the 21 kDa band was estimated to be 3.24 ± 0.25 (mean value ± SEM, $n = 7$), on account of densitometric records, following blot immunostaining with M-176 Ab, using fast muscle TC. Both size forms of αKAP were found to be present, in a similar ratio, in crude SR preparations from the pooled hind-limb muscles, except the soleus, of 7-day-old rabbits, i.e., from muscles destined to become fast muscles (not shown). In addition, we found that TC from mixed skeletal muscles, both rabbit diaphragm and human vastus l. [33], contained an admixture of the two size forms of αKAP, approximately in the ratio predicted by their mixed fiber composition (Fig. 4). We assessed that there was an overlapping distribution of both size forms of αKAP and of CaMKII β, γ, and δ subunits within the fast-twitch muscle SR.

Consistent with fluorescent images of cryostat sections of rabbit adductor muscle, showing a punctuate, "*triadic pattern*" of immunostaining, both for αKAP (using I-16 antibody) and for δCaMKII [8], αKAP peptide doublet and CaMKII-catalytically competent subunits were found to copurify with RyR1 and triadin monomer and polymers in Chaps-purified JFM (Fig. 3B). The estimated Bmax value for high-affinity [^{3}H]ryanodine binding to the same JFM preparation, using optimal binding assay conditions, was 35 pmol/mg

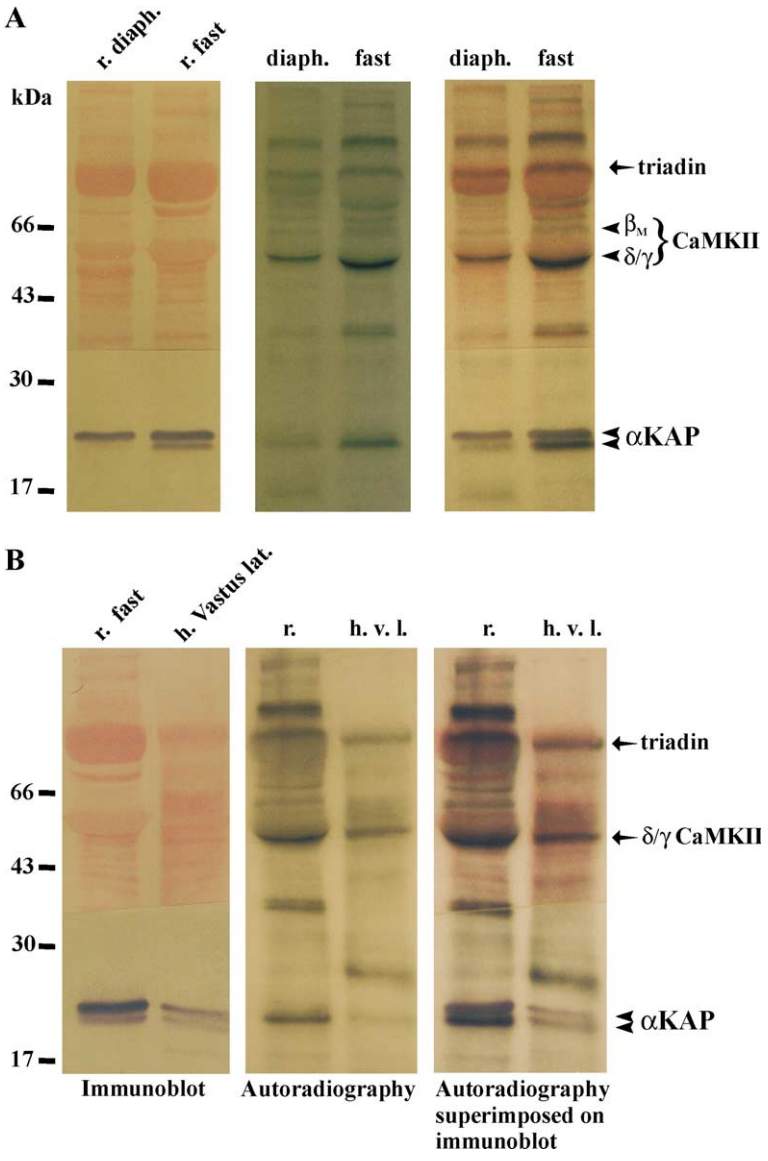

Fig. 4. Characterization of αKAP from various muscles by electrophoresis, Western blotting, and phosphorylation. (A) Immunoblots, autoradiographs of phosphorylated proteins, and superimposed images for the diaphragm (r. diaphr.) and adductor muscle (fast) of the rabbit. (B) Immunoblots, autoradiographs, and superimposed images for rabbit fast (r. fast) and human vastus l. (h.v. l.) in the same stated order. Sucrose-density purified TC were used for all muscles. TC were phosphorylated with [γ-^{32}P]ATP in standard phosphorylation assay medium (see Materials and methods) for 10 min. Blots after 10–15% SDS–PAGE (protein loading: 100 μg/lane) were exposed to autoradiographic films, and after being stained with Ponceau red, were cut above the 30 kDa molecular mass standard. The lower portion of blots was incubated with antibodies reactive to αKAP (M-176). There is an obvious overlapping of images at 21 kDa.

of protein, i.e., about three times the value in parental TC [22].

The lack of phosphorylation of αKAP by endogenous CaMKII in the isolated SR from rabbit soleus muscle appears to be consistent with differences in αKAP electrophoretic patterns in relation to fiber types. The demonstration that only the 21 kDa immunodetected peptide was phosphorylated by CaMKII was achieved in the experiments described in Fig. 4. The results in Fig. 5 show no effect of phosphorylation or dephosphorylation on the mobility of 21 kDa αKAP.

Stable integration of both size forms of αKAP into the CaMKII holoenzyme

The two size forms of αKAP co-purified with CaMKII γ/δ subunits by CaM-affinity chromatography of detergent extracts of TC from rabbit-fast-twitch muscle (Fig. 6A), using 0.25% Chaps in low ionic strength medium, as used for preferential solubilization of SR Ca^{2+}-pump membrane (also called "*free*") domain [21]. The tight inter-subunit association is supported by the results of co-immunoprecipitation experiments, using

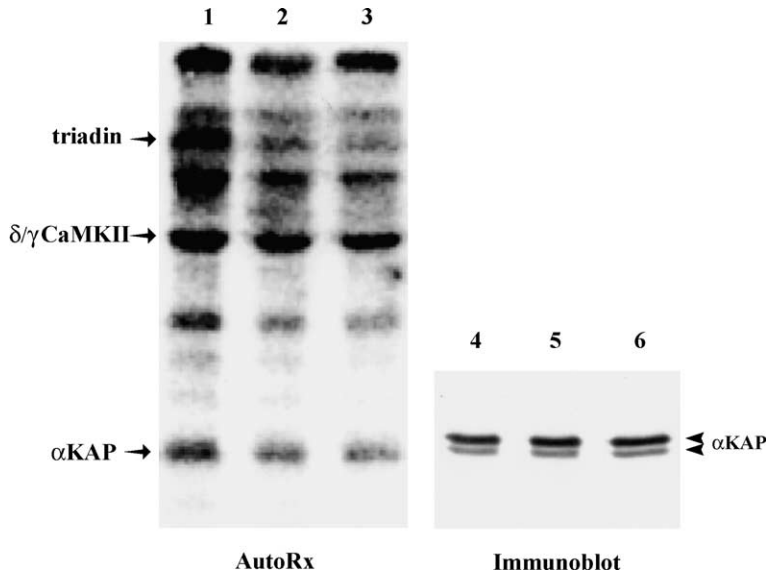

Fig. 5. Protein dephosphorylation by potato acid phosphatase vis à vis with Western blot analysis of αKAP peptide composition. Isolated TC from rabbit fast-twitch muscle were ^{32}P-labeled by incubating in standard assay medium at 0–4 °C for 10 min (lanes 1–3). Additional samples (lanes 4–6) were phosphorylated with cold ATP. CaMKII activity was inhibited by adding 2 μM staurosporin to the medium (lanes 1 and 4), followed by incubation with potato acid phosphatase (15 mU/50 μl) at 30 °C for 30 min (lanes 2 and 5), or 60 min (lanes 3 and 6). ^{32}P-labeled proteins, after 10–15% SDS–PAGE, were detected by autoradiography. Western blot analysis of 21 kDa αKAP electrophoretic mobility, using M-176 antibody, in phosphorylated (lane 4), and dephosphorylated, TC (lanes 5 and 6), after separation by 10–15% SDS–PAGE (protein loading: 35 μg/lane), is shown.

the purified kinase from EGTA eluates and antibodies against CaMKII δ subunit. These results are shown in Fig. 6B. Protein band detected in immunoprecipitates at 34 kDa was identified with protein-G, present as a minor contaminant and having the same mobility (see Materials and methods).

The degree of membrane solubilization with detergent Triton X-100 has provided important elements for the existence of specialized lipid microdomains (also termed rafts) in cell membranes (see [34]). Bayer et al. [6] reported that a combination of 2% Triton and a high ionic strength was required for effective solubilization of αKAP from rat skeletal muscle SR. We have found that a 1.4% Chaps 1 M NaCl solution was similarly effective and that under these conditions the tight association of both size forms with CaMKII full-length subunits was fully preserved. This was assessed by equilibrium centrifugation of detergent extracts on a linear (3–15%) sucrose-density gradient (i.e., using an experimental protocol patterned after the purification method reported by Lai et al. [35] for purifying RyR1). Thus, Western blot analysis of gradient fractions showed that both size forms of αKAP and CaMKII β, γ and δ subunits co-centrifuged to the same region (10–15% sucrose) of the gradient (not shown).

Computational prediction of two αKAP transcripts in mammalian skeletal muscle and supporting experimental evidence

Alternatively spliced variants of CaMKII α, δ, and γ, namely α_B, γ_A, and δ_B, contain a homologous 11-amino acid insert [4], as does αKAP [17,28]. Although not considered previously, it is rational to hypothesize that an alternatively spliced form of αKAP lacking the insert may exist.

We searched the GenBank database for sequences homologous to the 25-N-terminal amino acid sequence unique to αKAP. Besides the reported αKAP mRNA, we found three Expressed Sequence Tags (EST) obtained from *Mus musculus*, *Homo sapiens*, and *Bos taurus* cDNA libraries (Accession Nos. BB659027, AL520283, and BI849999, respectively) that lack the 33 nucleotides encoding for insert III of αKAP (for nomenclature, see [5]). The predicted proteins are 189 aa long, with a molecular mass 1232 Da inferior to the predicted molecular mass of αKAP.

To verify the existence of this alternatively spliced transcript we designed a set of primers to amplify the region of αKAP surrounding insert III. Forward primers are on the 75 nucleotides on the 5′ of the coding region unique to αKAP (insert I), while reverse primers are on the 3′ region shared also by full-length CaMKII α regions C–D) (Fig. 7A). Given the very high degree of conservation of this gene between mammals, we used primers based on the murine sequence to check by RT-PCR the expression of the two transcripts in rabbit muscle.

In rabbit soleus we could detect a single band, with a size matching the predicted 218 nucleotides of the mouse αKAP transcript fragment. However, in rabbit adductor magnus two bands were clearly visible, one with the same size as the previous fragment, the other about 30

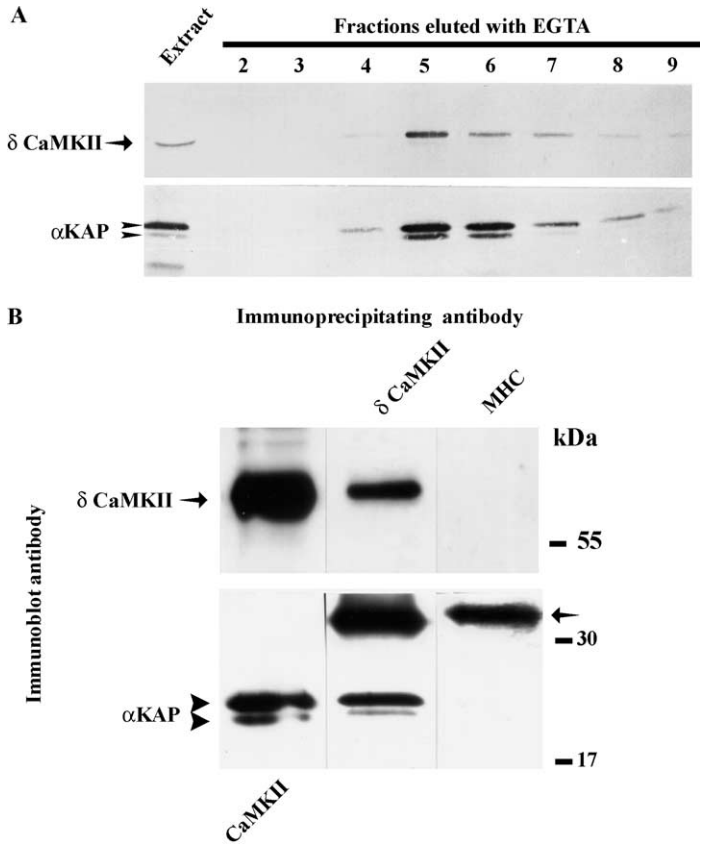

Fig. 6. Co-purification of CaMKII full-length subunits and αKAP peptide doublet from rabbit fast-twitch muscle SR. (A) CaMKII protein complex was purified from detergent (0.25% Chaps) extracts of TC by CaM-chromatography. About 5 mg of protein was loaded onto the column. Western blot analysis of fractions eluted by 2 mM EGTA, using antibodies reactive to δ CaMKII and to αKAP (M-176 Ab), is shown. (B) CaMKII protein complex purified by CaM-affinity chromatography was immunoprecipitated with anti-δ CaMKII (I-16) antibody. Immunoprecipitates bound to protein G–Agarose were solubilized in Laemmli's SDS buffer (see Materials and methods). Blots, after 10–15% SDS–PAGE, were probed with antibodies to δ CaMKII and to αKAP (M-176), as indicated. Arrow indicates protein-G. Positive controls were purified CaMKII. Negative controls were incubated with IgG against myosin heavy chain (MHC).

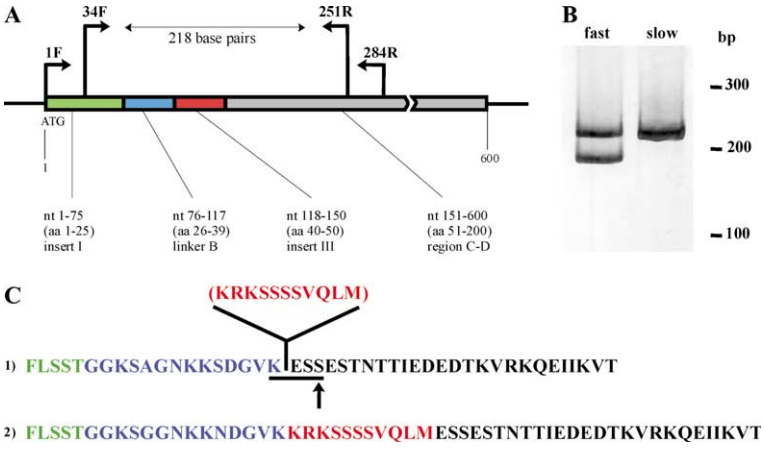

Fig. 7. (A) Structure of mouse αKAP cDNA. Key to colors: *green*, the 75 nucleotide sequence unique to αKAP, coding for the NH2-terminal hydrophobic domain (insert I, see [5]); *blue*, nucleotide sequence of linker B region, common to αKAP and CaMKII α; *red*, the 33 nucleotide, alternatively spliced sequence, coding for the 11-amino acid insert III; *gray*, the 3′ region common with CaMKII α regions C–D). Primers for the nested PCR are indicated by the arrows (see also Table 1). (B) RT-PCR products separated on a 12.5% polyacrylamide gel and visualized by silver staining. *Fast*: rabbit adductor m.; *Slow*: rabbit soleus. (C) (1) Sequence of the polypeptide encoded in rabbit fast-twitch muscle by the 185 bp band. In red, between brackets, the 11-amino acid insert III encoded by αKAP exon 2, missing in this transcript, but present in the 218 bp band. The putative KXXS phosphorylation consensus sequence for CaMKII is underlined. (2) Sequence of the corresponding region of mouse αKAP. Key to colors is, as in (A).

nucleotides shorter (Fig. 7B). Direct sequencing of the bands revealed that the longer fragment corresponds to αKAP, while the shorter corresponds to the transcript lacking the 33 bp-long (insert III) (Fig. 7C). Interestingly the absence of the 11-amino acid insert encoded by exon 2 creates a novel KXXS phosphorylation consensus site [4,36,37] that is present only in the shorter protein and not in αKAP (Fig. 7C).

Discussion

Our results make two important advancements toward molecular understanding of the function of CaMKII associated to junctional TC of the SR of mammalian skeletal muscle through anchoring protein αKAP.

First, we show that two size forms of αKAP, a common form (23 kDa) and a fast-twitch specific form (21 kDa), exist in native SR membranes from rabbit fast-twitch muscle, based on specific Western blotting after SDS–PAGE. We also demonstrate that both size forms are tightly associated to CaMKII catalytically competent β_M, γ, and δ subunits and are stably integrated into the holoenzyme, on account of CaM-affinity chromatography of detergent extracts and co-immunoprecipitation. This marks an important difference from CaMKII holoenzyme associated to slow-twitch muscle SR, in which only the common form of αKAP was immunodetected.

Second, we have approached here the problem of the genesis of the two size forms of αKAP, by computational analysis of the transcripts of the αCaMKII gene, and, directly, by amplifying and sequencing αKAP transcripts from mRNA isolated from representative fast- and slow-twitch skeletal muscles of adult rabbits. Primers were designed to amplify αKAP cDNA fragments containing part of insert I, linker B, the NLS-11-amino acid insert III and part of region C (see [5]). We demonstrate that rabbit fast-twitch skeletal muscle contains two types of mRNA transcripts that are generated by alternative splicing of the α gene. The transcript of larger size, appearing to be identical to the transcript present in slow-twitch skeletal muscle, was identified as the rabbit homologue of the cloned αKAP [17,28]. The transcript expressed exclusively in fast-twitch muscle was found to differ for the lack of the 33 bp insert III.

The two size forms of αKAP migrate on SDS–PAGE approximately at positions predicted by the presence or the absence of the 11-amino acid insert, having a calculated molecular size of 1232 Da. Despite correlations, warning about reliance on SDS–PAGE for determining molecular weights is suggested by the overestimated number that has been presented previously for rat αKAP [17], i.e., of 25 kDa, compared to a calculated molecular weight of 22.583 kDa [28]. We have found that improving the electrophoretic resolution of SR low molecular weight proteins, by increasing the length of the separating gel, has reduced the apparent M_r for the small size form of αKAP, from 23 kDa [7] to approximately 21 kDa. With these reservations, we propose that the two size variants differ only for the "*difference peptide*," as do variant A and variant B of brain αCaMKII [4,5].

Phosphorylation experiments identify αKAP-A, lacking the 11-amino acid insert, as the substrate of CaMKII in native SR membranes of rabbit fast-twitch muscle, implying that alternative splicing generates a CaMKII consensus sequence at a distinct domain. This was totally unforeseeable at the beginning of our studies [7]. We have tentatively identified KESS at the C-terminal end of linker-B region (see Fig. 7), as the minimal consensus sequence required for phosphorylation by CaMKII in situ, based on the fact that in several CaMKII substrates the basic residue on the N-terminal side of phosphorylation site is Lys and not Arg (for example, in caldesmon) [4,36,37]. In addition, a hydrophobic residue at P-5 is not an absolute requirement for CaMKII [4,36,37]. The predicted phosphorylation site is close to the site of insertion into membrane of N-terminal hydrophobic domain [17,28], suggesting that steric accessibility of the phosphorylation site might be regulated by "*boundary*" phospholipids tightly bound to the same domain.

In agreement with knowledge that the association of CaMKII to skeletal muscle membranes (including the nuclear membrane) is mainly governed by the hydrophobic properties of αKAP N-terminal end, and that the targeting information given by αKAP transmembrane domain overrides that given by NLS [4,38], we were unable to detect significant variations between fast-twitch and slow-twitch muscle in the distribution of CaMKII within the SR, following fractionation by isopycnic sucrose-density centrifugation. Nevertheless, we also have found that CaMKII was enriched in Chaps-purified JFM. That taken together with our own previous findings using immunofluorescence techniques [8] is in support of a preferential association of CaMKII to the junctional membrane domain of the SR in this type of muscle. Our findings with rabbit fast adductor muscle can be compared and contrasted with a recent report on transgenic skeletal muscle fibers of rat slow soleus, in which using hemagglutinin-tagged αKAP, this was localized almost exclusively to longitudinal SR [38]. Although undue reliance on a single experimental method might provide wrong leads, underlying this finding is the knowledge that CaMKII-mediated modulation of SR Ca^{2+}-transport system is dictated by the expression of specific protein substrates (SERCA2a and phospholamban), present exclusively in slow-twitch muscle Ca^{2+}-pump membrane (both longitudinal SR and the extra-junctional portion of TC).

The present state of comparatively poor molecular knowledge of CaMKII action in fast-twitch muscle SR is witnessed by the fact that, after almost a decade since the discovery that CaMKII modulates the native Ca^{2+}-release channel (i.e., in TC) [11], and despite subsequent phosphorylation studies on isolated TC from rabbit fast-twitch muscle [7,8,12], there is still a wide divergence of opinions regarding the functional correlates of CaMKII-mediated phosphorylation at the single Ca^{2+}-channel level [39], as well as over whether relevant phosphorylation sites are on RyR1 or on triadin [8]. Triadin ability to interact structurally and functionally with RyR1 is now well documented [40].

The regulative features and ability of CaMKII to act as a transducer for the frequency, amplitude, and duration of intracellular Ca^{2+} signals [1,4] enable it not only to respond to brief Ca^{2+} transients but also to keep memory of repetitive muscle activation (e.g., post-tetanic potentiation of twitch force), as found in mammalian fast-twitch muscle [13,14]. Therefore, the demonstration that alternative splicing regulates the phosphorylation of αKAP in the same type of muscle could have important functional implications. It will also be interesting to elucidate whether alternative splicing is regulated by the specific motor innervation to the muscle, i.e., by the specific frequency and pattern of firing of α motoneurons [41].

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