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Phosphorylation of Anchoring Protein by Calmodulin Protein Kinase Associated to the Sarcoplasmic Reticulum of Rabbit Fast-Twitch Muscle

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Regulatory phosphorylation of phospholamban and of SR Ca^{2+}-ATPase SERCA2a isoform by endogenous CaM-K II in slow-twitch skeletal and cardiac sarcoplasmic reticulum (SR) is well documented, but much less is known of the exact functional role of CaM K II in fast-twitch muscle SR. Recently, it was shown that RNA splicing of brain-specific α CaM K II, gives rise to a truncated protein (αKAP), consisting mainly of the association domain, serving to anchor CaM K II to SR membrane in rat skeletal muscle [Bayer, K.-U., *et al.* (1998) *EMBO J.* 19, 5598–5605]. In the present study, we searched for the presence of αKAP in sucrose-density purified SR membrane fractions from representative fast-twitch and slow-twitch limb muscles, both of the rabbit and the rat, using immunoblot techniques and antibody directed against the association domain of α CaM K II. Putative αKAP was immunodetected as a 23-kDa electrophoretic component on SDS–PAGE of the isolated SR from fast-twitch but not from slow-twitch muscle, and was further identified as a specific substrate of endogenous CaM K II, in the rabbit. Immunodetected, ^{32}P-labeled, non-calmodulin binding protein, behaved as a single 23-kDa protein species under several electrophoretic conditions. The 23-kDa protein, with defined properties, was isolated as a complex with 60-kDa δ CaM K II isoform, by sucrose-density sedimentation analysis. Moreover, we show here that putative αKAP, in spite of its inability to bind CaM in ligand blot overlay, co-eluted with δ CaM K II from CaM-affinity columns. That raises the question of whether CaM K II-mediated phosphorylation of αKAP and triadin together might be involved in a molecular signaling pathway important for SR Ca^{2+}-release in fast-twitch muscle SR.

Key Words: **skeletal muscle; sarcoplasmic reticulum; calmodulin protein kinase; A-kinase anchoring proteins.**

Abbreviations used: CaM K II, calmodulin protein kinase; αKAP, αCaM K II association protein; PLB, phospholamban; RyR1, Ca^{2+}-release channel/ryanodine receptor, skeletal isoform; SERCA, sarcoendoplasmic reticulum Ca^{2+}-ATPase; SR, sarcoplasmic reticulum.

The sarcoplasmic reticulum (SR) plays a central role in regulation of the contraction-relaxation cycle in both fast-twitch and slow-twitch muscles. There are, however, major differences between Ca^{2+}-release and Ca^{2+}-transport in these muscles (1), affecting the amplitude and time-course of Ca^{2+}-transients (2). Regarding Ca^{2+}-transport, these are manifested in the special regulatory features of the transport system in slow-twitch muscle SR, i.e., similar to those of cardiac muscle SR (3, 4). Protein phosphorylation is a primary mean by which the slow-twitch isoform of SR Ca^{2+}-ATPase (SERCA2) is highly regulated by calmodulin (CaM) and Ca^{2+}-activated protein kinase II (CaM K II), either directly, i.e., through phosphorylation of SERCA2 (5) at Ser-38 (6), or indirectly, through phosphorylation of regulatory protein phospholamban (PLB) at Thr-17 (7).

Targeting of cyclic AMP-activated protein kinase A (PKA) to specific subcellular compartments occurs through association to distinct members of A-kinase anchoring proteins (AKAPs) (8). Similarly, a study on rat limb total muscles, found that targeting to SR membrane of the several CaM K II isoforms, including skeletal muscle-specific βM isoform, involves binding to anchor protein αKAP with a calculated molecular weight of 25 kDa (9), and which was identified as an alternative, non-kinase product of brain specific α CaM kinase (9, 10). The physiological relevance of this mechanism, in the case of rat slow-twitch muscle, in particular, is still rather obscure, however. That mainly because this type of muscle, unlike homologous limb muscles of larger mammals, e.g. the rabbit, was found to be deficient in PLB, a fiber type-specific target of CaM K II in skeletal muscle SR (19). Early biochemical-functional studies on CaM K II associated

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to the SR of rabbit fast-twitch muscle (11–19), referred to also as 60-kDa CaM K II isoform focused on its differential ability to phosphorylate RyR1-associated proteins in such muscle, among which triadin (17–19).

In an attempt to demonstrate the presence of αKAP in sucrose-density purified SR membrane fractions from rabbit fast-twitch muscle, we used Western blot techniques and specific antibodies to brain α CaM K II. Results reported here show that antibody recognized a single protein of 23 kDa on SDS–PAGE, and that this protein, probably homologous to rat αKAP, acts as a specific substrate of anchored CaM K II. Portions of this work have been presented in poster form at the 29th European Muscle Conference, Berlin, Germany.

MATERIALS AND METHODS

All chemicals were analytical grade. Molecular mass standards for SDS–PAGE were from BDH Lab. Supplies (Poole, England). $^{45}CaCl_2$ was from Radiochemical Centre (Amersham, England).

Preparation of skeletal muscle SR. SR vesicles were isolated from homogenates of predominantly fast-twitch, or slow-twitch skeletal muscles from the hind-legs of New Zealand male adult rabbits or Wistar rats. In the case of rats, muscles used were the tibialis anterior and soleus, respectively. The isolated SR was purified by isopycnic sucrose-density centrifugation, using the method of Saito *et al.* (20), with slight modifications (21), to yield four distinct fractions (labeled R1 to R4, from top to bottom of gradient). Membrane fractions from the sucrose-density gradient were stored at −80°C, until used. Protein concentration was determined by the Folin reaction (22), using BSA (Boehringer, Mannheim, Germany) as a standard.

Solubilization of SR with Nonidet-P40. Sucrose-density purified SR membranes (generally R2, or R3) were incubated, at 3–4 mg protein/ml for 30 min at 37° with 10 mM Tris, pH 8.3, 1 mM EGTA, and centrifuged for 60 min at 120,000*g*, as described by Tuana and MacLennan (14). Following the same procedure, pellets were then resuspended in 2 ml of 10 mM Tris, 1 mM histidine, pH 8.0 (buffer A). After addition of 140 μl of 10% NP-40 (final concentration 0.7%), the mixture was incubated for 15 min on ice and then centrifuged for 90 min at 150,000*g* to yield the final extract.

Isolation and purification of CaM K II-anchoring protein complex. NP-40 extracts of SR R2 fraction from rabbit fast-twitch muscles, were loaded onto linear gradients of 5–30% sucrose in buffer A containing 0.7% NP-40, and then centrifuged at 30,000 RPM for 17 h, using a Beckman SW 40 rotor. Gradient fractions (0.5 ml) were collected from the top of the gradient, and protein samples (100 μl) were analyzed by SDS–PAGE, immunoblotting and autoradiography, after phosphorylation with [γ-^{32}P]ATP (see below).

CaM-Sepharose affinity chromatography of NP-40 extracts was carried out, essentially as described by Tuana and MacLennan (14).

Phosphorylation. Experimental assay conditions for endogenous CaM K II activity were reported previously (17–19), without or with 1 μM CaM. The concentrations of [γ-^{32}P]ATP (specific radioactivity 0.10 Ci/mmol) (NEN, Du Pont De Nemours, Bad Homburg, Germany) was 400 μM. The reaction was quenched by solubilizing in SDS–buffer. Electrophoretic SDS-gels after being dried, were analyzed by autoradiograpy (16 h exposure), using Hyperfilm (Amersham), or by a Model GS-250 Molecular Imager (Bio-Rad Laboratories, Hercules, CA), using a β-particles sensitive screen.

Digestion of intact TC vesicles with chymotrypsin. TC suspended in 0.3 M sucrose, 5 mM imidazole, pH 7.4, were digested with chymotrypsin (protease to protein ratio 1:200) for 5 or 10 min at 20°C. The reaction was stopped with 20 mM PMSF and SDS solubilization buffer.

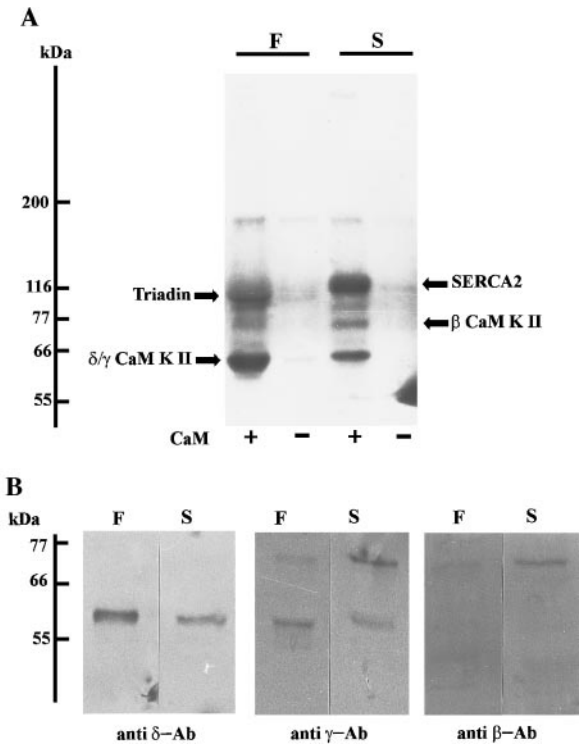

FIG. 1. Comparison between isolated TC from rabbit fast-twitch and slow-twitch muscle, regarding the pattern of protein phosphorylation. (A) Autoradiography. Phosphorylation of isolated TC from rabbit fast-twitch (F) and slow-twitch muscle (S) was, as described under Materials and Methods, without or with 1 μM CaM. Incubation time was 5 min. ^{32}P-labelled proteins were detected by autoradiography after 5–10% SDS–PAGE. Fifty micrograms of protein was loaded per gel. Molecular mass standards are indicated on the left. Position of individual proteins is marked by arrows. For abbreviations of names, see text. (B) Immunoblotting. Blots after 5–10% SDS–PAGE (20 μg/gel), were incubated with polyclonal antibody to δ and to γ CaM K II isoform, respectively, or with monoclonal antibody to β isoform, as indicated. The δ and γ isoforms are localized to 60 kDa, and the β CaM K II isoform to 72 kDa. The polyclonal antibody to γ CaM K II is shown to be cross-reactive with the β isoform.

Gel electrophoresis, immunoblotting, and ligand overlays. One-dimensional SDS–PAGE was carried out according to Laemmli (23), as reported in the legends to the figures. Two-dimensional electrophoresis, in the presence of 0.1 mM EGTA in the first dimension, and of 1 mM $CaCl_2$ in the second dimension, was carried out as described by Damiani and Margreth (24). Slab gels were stained with Coomassie blue or with silver nitrate (25). Blots of proteins transferred onto nitrocellulose were probed with goat polyclonal antibody to α, δ and γ isoforms of CaM K II (Santa Cruz, CA) (1 μg/ml) (10, 21, 26), or with mouse monoclonal antibody to β isoform (Cbβ-1, GIBCO BRL, Life Technologies) (10, 26), as described previously (19). The anti-α CaM K II antibody (C-17) was raised against a peptide mapping at the carboxyl terminus of α CaM K II of mouse origin. Antibody binding was detected by immunoenzymic staining (19). ^{45}Ca overlay of blots was done by previously described methods (21). Overlay of blots with biotinylated CaM (Biochemical Technologies Inc., Stoughton, MA) was carried out, as described previously (17).

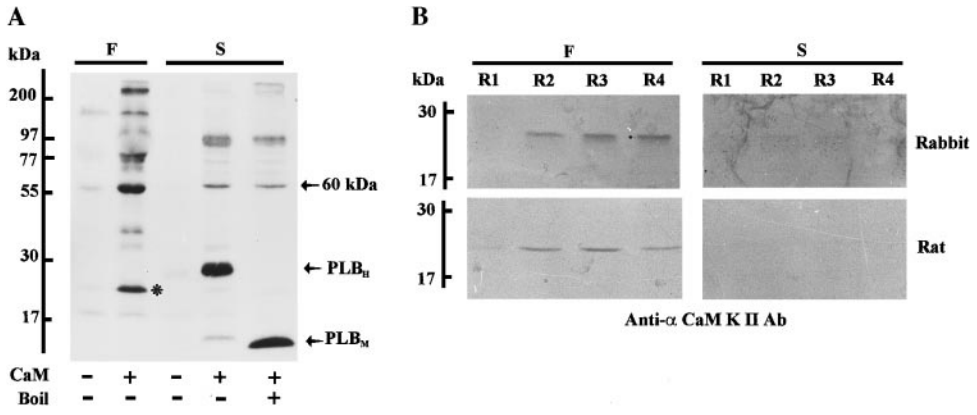

FIG. 2. CaM K II-mediated phosphorylation of phospholamban versus phosphorylation of 23-kDa protein in the isolated SR and differential immunoreactivity of 23 kDa in slow-twitch compared to fast-twitch muscle. (A) Autoradiogram. Phosphorylation of TC was performed as described in the legend to Fig. 1. SR proteins were resolved using 10–15% SDS–PAGE, without and with previous boiling. PLB_H, pentameric PLB; PLB_M, monomeric PLB. Asterisk indicates 23-kDa protein. (B) Immunoblotting. Isopycnic centrifugation in a sucrose step gradient of SR membranes from fast-twitch (F) or slow-twitch (S) muscles of the rabbit and rat, gave four distinct fractions, as described. Junctional TC fractionate to R4, longitudinal SR vesicles to R2, and transverse tubule fragments, to R1. Protein (50 μg/gel) was resolved by 10–15% SDS–PAGE. Blots were incubated with polyclonal antibodies to the α isoform of CaM K II. Molecular mass standards are indicated on the left. Position of individual proteins is indicated.

RESULTS AND DISCUSSION

There are differences between limb fast-twitch and slow-twitch muscles of the rabbit in the pattern of protein phosphorylation of the isolated SR by endogenous CaM K II, as reported recently (19, 26). To summarize these briefly, in slow-twitch muscle SR CaM K II phosphorylates the SERCA2a Ca^{2+}-ATPase isoform (Fig. 1A), in addition to PLB (Fig. 2A). The lack of these proteins in fast-twitch muscle SR accounts for the early observation that the action of CaM K II, in this case, seems to be restricted to junctional SR-specific proteins, e.g., triadin (17–19, 26). In this respect, quantitative differences between fast-twitch and slow-twitch muscles, appear to be amplified, using sucrose-density purified junctional TC (Fig. 1A), given also the very high degree of purity of TC vesicles, when isolated from rabbit fast muscle. As far as SR proteins of low molecular weight acting as specific substrates of endogenous CaM K II, fast-twitch muscle SR appears to be characterized for the presence of an electrophoretic component, immunologically unrelated to PLB (26), and having a faster mobility than PLB pentamer (Fig. 2A).

The results in Figs. 1 and 2 outline significant differences between fast-twitch and slow-twitch muscle SR,

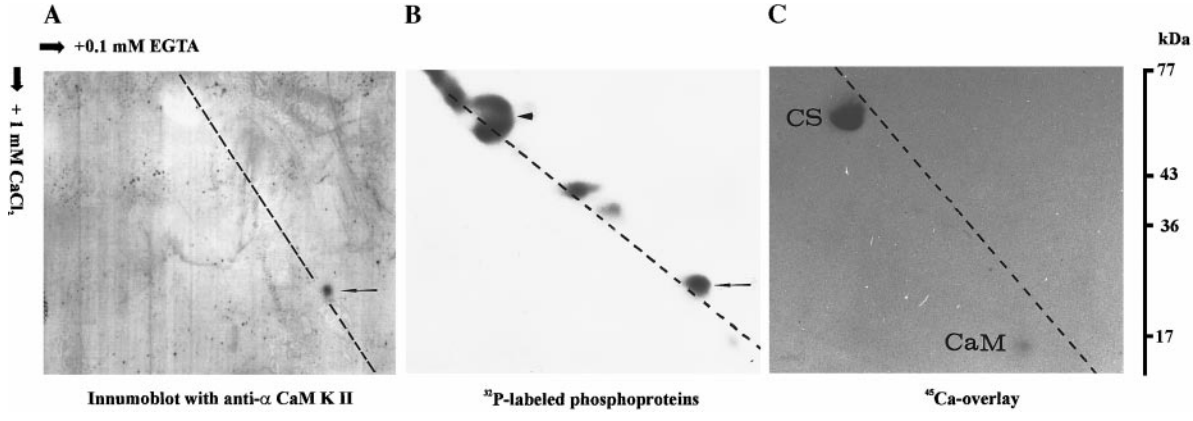

FIG. 3. Electrophoretic separation of 23-kDa SR protein from calmodulin. Two-dimensional electrophoresis of unincubated rabbit fast-twitch muscle TC or following incubation with [γ-^{32}P]ATP in CaM K II-assay medium, was carried out in the presence of 0.1 mM EGTA in the first dimension and of 1 mM $CaCl_2$ in the second dimension in gels loaded with 100 μg (see Materials and Methods). Dashed lines mark the position of diagonals. (A) Western blotting with antibody to α CaM K II. Arrow indicates the 23-kDa protein. (B) Autoradiogram. Arrowhead and arrow indicate 60-kDa CaM K II and 23-kDa phosphoprotein, respectively. (C) Autoradiogram of blots, after overlay with ^{45}Ca, as described under Materials and Methods. CS, calsequestrin; CaM, calmodulin.

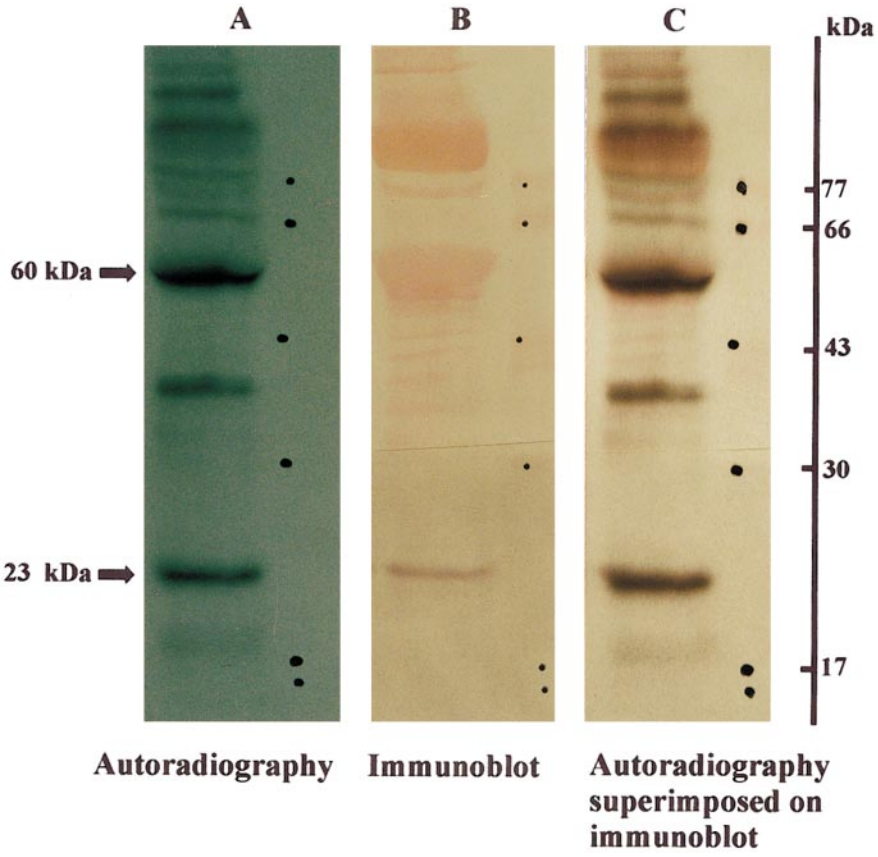

FIG. 4. Protein homogeneity of 23-kDa electrophoretic band, on the basis of ^{32}P-labeling and of immunoblotting with antibody to α CaM K II. Rabbit fast-twitch muscle TC were phosphorylated with [γ-^{32}P]ATP, using the standard experimental assay conditions for endogenous CaM K II. Samples (100 μg) were analyzed by 10–15% SDS–PAGE and transferred onto nitrocellulose. The blot, after ^{32}P-autoradiography (A), was stained with Ponceau red and cut above the 30-kDa molecular mass standard. The lower portion of the blot was incubated with anti-α CaM K II antibody (B). In (C) the ^{32}P-autoradiography is superimposed on immunoblot.

concerning also the pattern of isoform composition of CaM K II, as underlined by differences in the level of ^{32}P radioactivity incorporated into 60 kDa and 72 kDa, the protein bands, corresponding to self-phosphorylated γ/δ isoforms, and β CaM K II isoform, respectively (10, 26). Identification of the several CaM K II isoforms on blots relied on the combined use of polyclonal antibodies to γ and to δ CaM K II (19, 26) and of a specific monoclonal antibody to β CaM K II (10, 26). The preferential expression of β CaM K II in slow-twitch muscle SR (Fig. 1B), had been already observed (26). The 72-kDa polypetide found to be cross-reactive with antibody to γ CaM K II (19) appears to be identical to peptide stained by anti β CaM K II antibody. While it is a well acquired notion that CaM K II isoform associated to rabbit predominantly fast-twitch SR is predominantly a 60-kDa protein isoform (11–19), and thus in agreement with the evidence shown here and in a recent report (26), the only reported, immunological evidence that δ and β CaM K II isoforms are present in equal amounts in skeletal muscle SR, comes from a study in the rat, in which the SR was isolated from total limb muscles (10).

The same study found that an alternative, nonkinase product of a gene within the muscle α CaM K II gene, consisting mainly of the associative domain (αKAP), anchors CaM K II to rat skeletal muscle SR through a structural interaction in which a skeletal muscle specific, β_M CaM K II isoform, was suggested to be involved (10). To try to get some clue as to whether the protein level of expression of anchor protein might differ between fast-twitch and slow-twitch muscle SR, as well as depending on the animal species, we tested sucrose-density purified SR fractions from the several types of muscles, using Western blot techniques and antibody directed against the association domain of brain α CaM K II, on account of the structural interelatedness between such domain and skeletal muscle αKAP (9, 10). The results of these experiments are shown in Fig. 2B. As predicted, this antibody specifically recognized in rabbit and rat fast-twitch muscle a protein electrophoretic component of 23 kDa, in good agreement with the calculated molecular size of αKAP of 25 kDa (9), and which displayed a distribution between the four membrane fractions from the isopycnic

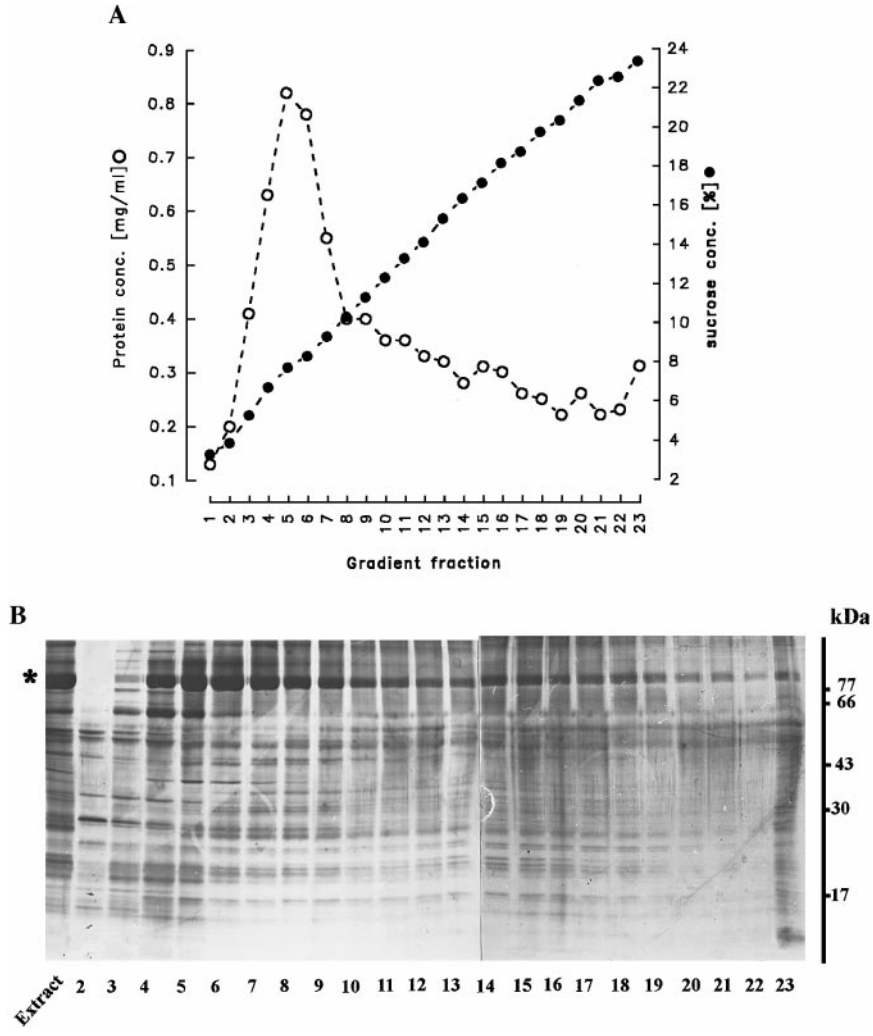

FIG. 5. Sedimentation profile of detergent solubilized SR proteins, following centrifugation through a linear sucrose-density gradient. (A) Nonidet-40 extracts of SR membranes (R2) from rabbit fast-twitch muscle, were centrifuged overnight on a linear 5–30% sucrose sedimentation gradient (see Materials and Methods). Following fractionation (0.5 ml/fraction) of the gradient, aliquots (50 μl) were taken for determination of protein concentration. (B) Protein was resolved by 10–15% SDS–PAGE, and stained with silver nitrate. Position of SR Ca^{2+}-ATPase major protein is indicated by asterisk.

sucrose-density centrifugation gradient, that are those expected from a SR specific protein, ubiquitously present in free and in junctional SR (10). The negative evidence that we obtained using the corresponding membrane fractions from slow-twitch muscles of both species, was an unexpected finding (Fig. 2B). The differential distribution between fast-twitch and slow-twitch muscle SR of 23-kDa immunostained protein and which, therefore, resembled closely the distribution of ^{32}P-phosphoprotein having an electrophoretic mobility also similar, identified in rabbit fast-twitch muscle SR as a specific target of CaM K II, prompted us to investigate more closely the actual relationship between the two proteins.

The 23-kDa protein reported here, has many properties in common with an earlier described SR protein substrate of endogenous CaM K II, in early studies using the isolated SR from predominantly fast muscle of the rabbit and referred to as 20-kDa protein (11–14). Thus, we similarly found that the 23-kDa protein specific to fast-twitch muscle SR exhibited a slower rate of phosphorylation, compared to the rate of self-phosphorylation of 60 CaM K II (not shown), and also that it did not to bind CaM using overlay techniques (see Fig. 7C). Although it was early proposed that 20-kDa phosphoprotein of rabbit fast-twitch muscle SR might be calmodulin (11), that remained unsupported. Using junctional TC, to which significant amounts of endogenous CaM remain bound (18), we were able to separate CaM from 23-kDa protein by 2D SDS–electrophoretic analysis, in the presence of EGTA in the first dimension, and of $CaCl_2$ in the second dimension (Fig. 3). On the contrary, we were unable to resolve the 23-kDa phosphoprotein from αKAP, as iden-

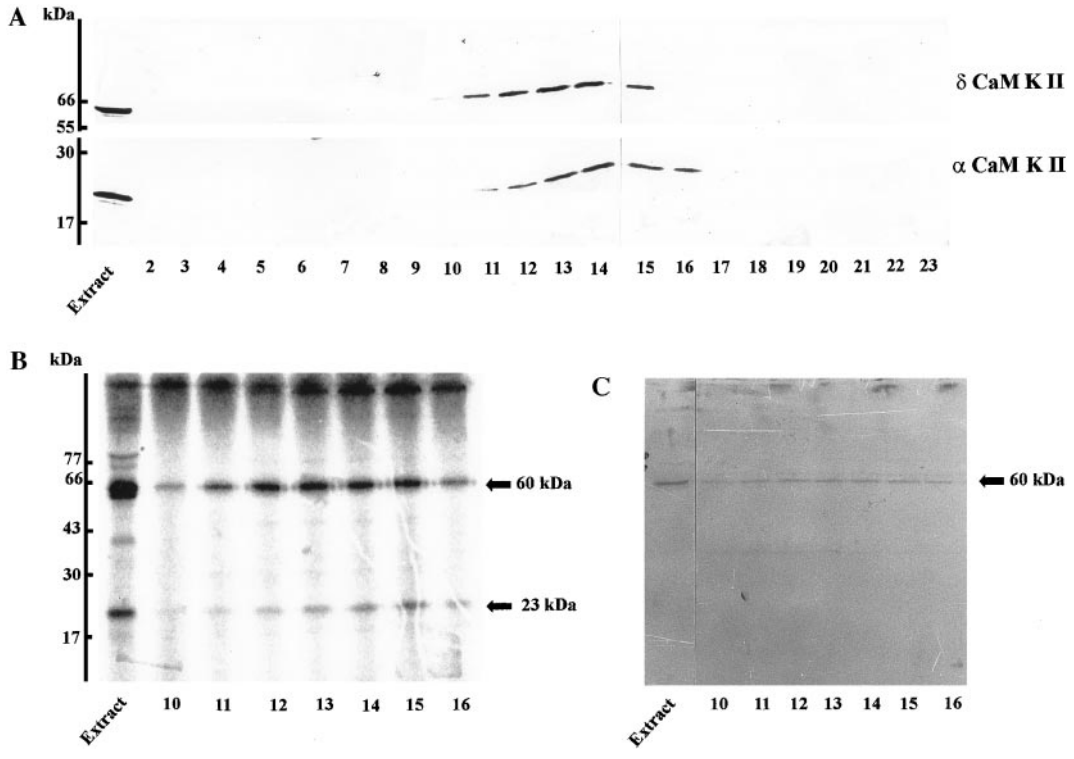

FIG. 6. Sedimentation profile of CaM K II-23 kDa protein complex. (A) Aliquots (100 μl), of sucrose sedimentation gradient fractions were subjected to 10–15% SDS–PAGE. Blots were incubated with specific polyclonal antibodies to δ CaM K II and α CaM K II, as indicated. Molecular mass standards are indicated on the left. (B) Additional aliquots of fractions 10–16 were phosphorylated with [γ-^{32}P]ATP, in standard assay medium before being subjected to SDS–PAGE. ^{32}P-labeled proteins were detected by exposing dried gels onto β particle-sensitive screens for 180 min. Screens were scanned using a Bio-Rad GS-250 molecular imager, and images were collected and analyzed using Phosphor Analyst/PC Image analysis software (Bio-Rad). Original autoradiograms were printed using a digital Mitsubishi (CP-D1E) color printer. (C) Blots of SDS-gels, were overlayed with biotinylated CaM, at pCa 4. Molecular mass standards are indicated on the left.

tified by immunoblotting with antibody to α CaM K II, using the same electrophoretic system (Fig. 3A), or else employing a Bio Rad Protean apparatus and 16-cm long slab gels (Fig. 4). These findings, therefore, argued that the 23-kDa phosphoprotein and putative αKAP may be indeed the same protein.

Consistent with the proposed membrane topology, at the cytoplasmic surface of SR membrane, of CaM K II anchor protein complex in rat skeletal muscle (10), we have found that δ CaM K II (i.e., the apparently predominant CaM K II isoform in rabbit fast-twitch muscle SR), and putative αKAP, were similarly highly sensitive to mild proteolytic digestion by chymotrypsin, using intact SR vesicles (data not shown).

To assess that, in fast-twitch muscle SR, CaM K II is tightly associated with putative αKAP, as implied by the previous study in rat skeletal muscle SR (10), in complementary experiments we tried to isolate such protein complex from Nonidet-40 extracts (see Methods), following centrifugation through a 5–30% linear sucrose gradient. Proteins from the sucrose gradient fractions were separated by SDS–PAGE, and were detected by silver staining (Fig. 5B), in combination with specific Western blotting (Fig. 6A) and ligand blot overlay with biotinylated CaM (Fig. 6C). Clearly, sucrose gradient sedimentation of the detergent extract separated the bulk of protein, consisting mainly of SR Ca^{2+}-ATPase of about 100 kDa Mr and which was found to peak in fraction 6 (Fig. 5B), from 60-kDa CaM K II and 23-kDa putative αKAP, displaying an identical sedimentation pattern, and which appeared to be selectively associated to fractions 10–16 (Fig. 6). To follow-up the distribution of CaM K II between gradient fractions, the protein kinase was identified by specific immunoblotting, using polyclonal antibody to δ CaM K II (Fig. 6A) as well as by ligand blot overlay with biotinylated CaM (Fig. 6C). As shown in Fig. 6B, the pattern of distribution of 60 kDa and 23 kDa ^{32}P-labeled phosphoproteins between fractions, following incubation in the presence of Ca^{2+} and CaM, was found to be the same as that observed using Western blot techniques to identify these proteins.

In additional experiments, we used CaM-Sepharose affinity chromatography to purify CaM K II from Nonidet-P40 extracts of SR membranes of rabbit and rat fast-twitch muscle, at the light of an early study (14). We found that CaM K II from either kind of muscle, bound to column in the presence of Ca^{2+}, and

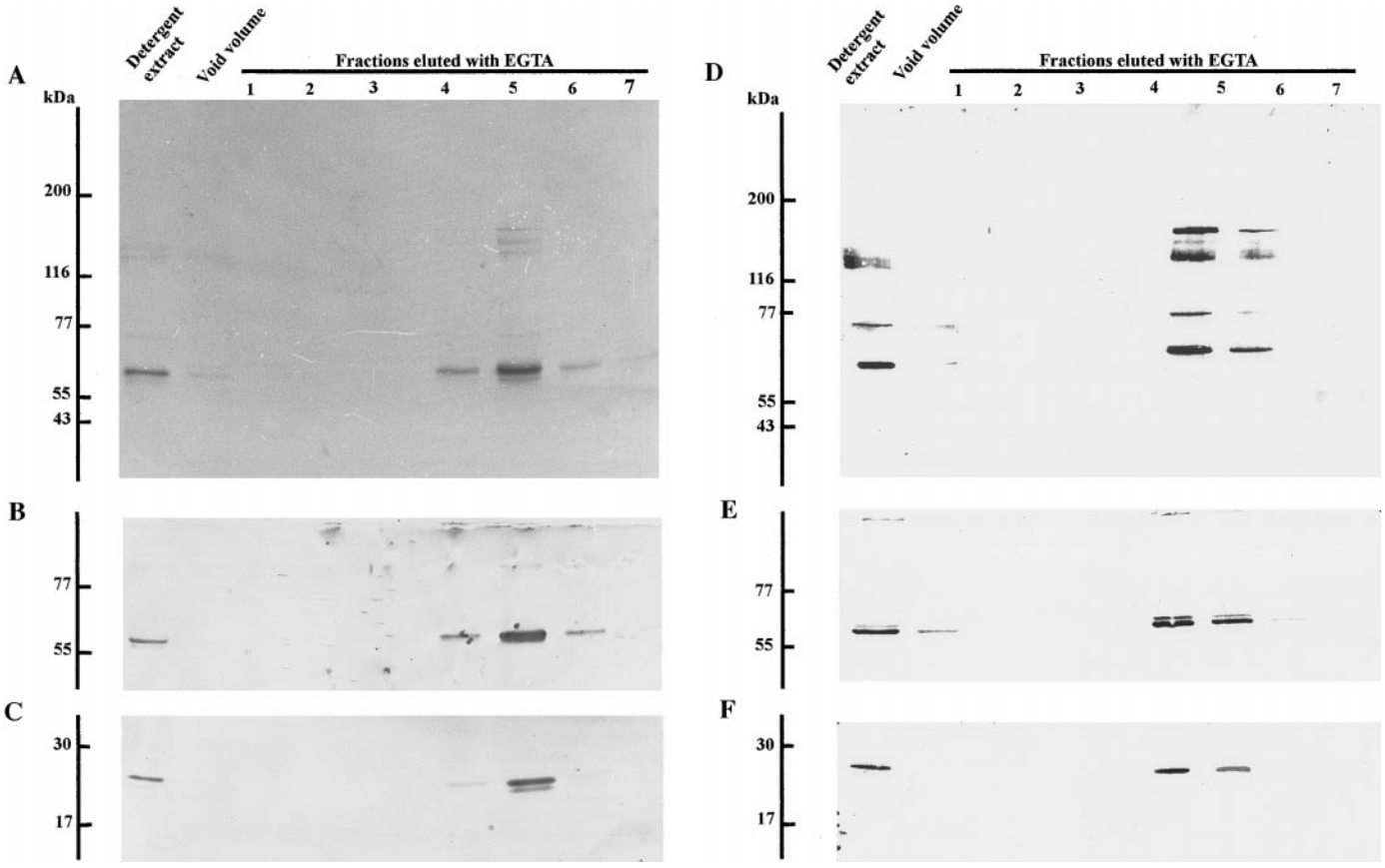

FIG. 7. Elution profile of δ CaM protein kinase and of 23-kDa protein from CaM-Sepharose affinity column. Approximately 5 mg protein of sucrose-density purified (R3 fraction), detergent-solubilized SR from fast-twitch muscle of the rabbit and the rat was loaded onto CaM-Sepharose column equilibrated with 1 mM $CaCl_2$. Proteins eluted with 2 mM EGTA, were resolved using 5–10% (A, D) or 10–15% SDS–PAGE (B, C, E, F), respectively. Blots either were overlayed with biotinylated CaM (A, D), or were immunostained with antibody to δ (B, E) or to α CaM K II, respectively (C, F). In A and D, the additional CaM-binding proteins of molecular mass ranging from 130 to140 kDa, and found to be prominent with rat fast-twitch muscle, are identifiable with plasma membrane Ca^{2+}-ATPase protein bands, based on previous work [33], and are explained with the presence of a varying amount of contaminant TT in the particular membrane fraction.

was similarly eluted with EGTA, as detected by ligand blot overlay with biotinylated CaM (Figs. 7A and 7D), in addition to immunoblotting with anti δ CaM K II antibody (Figs. 7B and 7E). The high sensitivity of CaM-overlay technique, allowed also to detect the presence of contaminant PMCA, appearing to co-elute with δ CaM K II, using detergent extracts of rat SR, but much less so, using SR membranes from rabbit fast-twitch muscle with a higher degree of purity (Fig. 7). The results obtained by immunoblotting with antibody to α CaM K II and which are presented in the same Fig. 7 (C and F), provide evidence that regardless of the extent of contamination with PMCA, putative αKAP, shown not to bind CaM on ligand blot overlay (Fig. 6C), co-eluted with CaM K II. That is an additional important element in support of the interpretation that αKAP forms a stable complex with δ CaM K II isoform in rabbit fast-twitch muscle SR, i.e., not dissociated during CaM K II purification procedure. The real identity of αKAP with 23-kDa phosphoprotein, is corroborated by the analogous evidence that ^{32}P-labeled protein co-purified with self-phosphorylated CaM K II (Fig. 8).

Conclusively, the results reported here, seem to be able to put together a number of previous observations into a more coherent picture. The perhaps most original observation in the present study, is that putative αKAP, is a specific substrate of anchored CaM K II in native SR vesicles from rabbit fast-twitch muscle, as well as in the isolated protein complex. The importance of this observation finding appears to be strengthened by the identification of a specific consensus sequence for CaM K II (R-X-X-S) (27), between amino acid residues 41 and 44 of αKAP deduced primary structure, i.e., near to αKAP specific sequence (amino acids 1 to 25), corresponding to the short intramembrane segment of the protein molecule (9).

The hypothesis that regulation of the localization of protein kinases to specific cell subcompartments might be a function of targeting of different members of anchoring proteins, has grown largely from knowledge of AKAPs. Despite extensive structural heterogeneity,

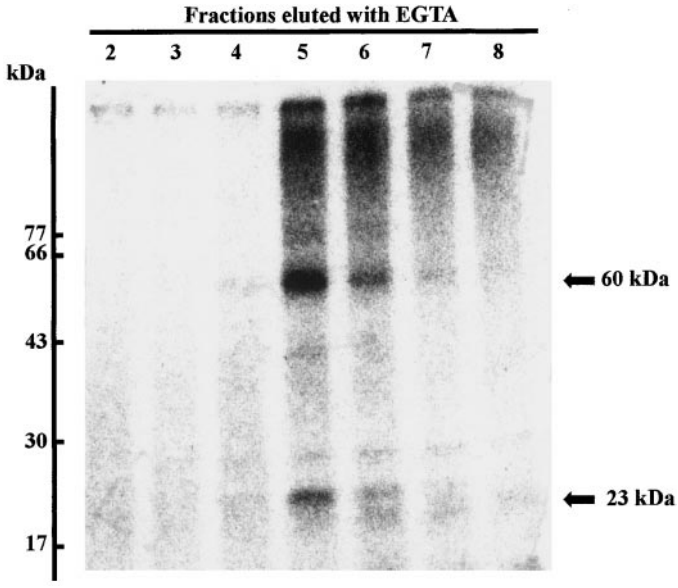

FIG. 8. Co-elution of ^{32}P-labeled CaM K II and 23-kDa protein from CaM-Sepharose affinity column. Detergent-solubilized SR protein, following incubation for 10 min with [γ-^{32}P]ATP in the presence of 100 μM Ca^{2+} and 1 μM CaM, was loaded onto CaM-Sepharose column. Aliquots (100 μl) of fractions, eluted with 2 mM EGTA, were analyzed by using 10–15% SDS–PAGE. ^{32}P-labeled proteins were detected, as described in the legend to Fig. 7B, by exposing dried gels onto β particle-sensitive screens, and scanning screens by a Bio-Rad GS-250 molecular imager, equipped with Phosphor Analyst/PC Image analysis software (Bio-Rad). Molecular mass standards are indicated on the left. The position of individual proteins is indicated.

AKAPs can be grouped together into the same family, because of a common binding site for the regulatory subunit (RII) of PKA (28, 29). For at least one member of this family (AKAP79), membrane targeting was found to be regulated by PKC phosphorylation (30).

Our present experimental findings, in principle, do not deny the possibility that αKAP gene within the α CaM K II gene in skeletal muscle fibers might be expressed only in fast-twitch fibers. However, considering that αKAP consists mainly of the association domain of the α subunit, and that the NH_2-terminal region of this domain is the least conserved sequence of the different CaM K II isoforms, being referred to as the variable region (27), a perhaps more plausible hypothesis is that distinct molecular variants of this protein, with a low extent of antigenic homology might be expressed in skeletal muscle SR in relation to specific fiber types.

The Ca^{2+} and CaM dependent autophosphorylation of CaM K II at Thr-286 by which the enzyme becomes converted to an autonomous, Ca^{2+}-independent form (27), is expected to prolong the effects of depolarization-induced Ca^{2+}-transients in skeletal muscle fibers (31). It would seem that generation of autonomy is not the same for the several CaM K II isoforms (31), and we have found ((26) and present work), that fast-twitch muscle SR differs from slow-twitch muscle SR in containing γ and δ isoforms in much higher amounts than β CaM K II isoform. Since rabbit fast-twitch muscle SR is shown here also to differ in containing a phosphorylated isoform of αKAP, the number of biological variables involved is enormous. It may be that αKAP has to be phosphorylated for phosphorylation of triadin to occur in junctional TC of fast twitch muscle, since in experiments in which the effects on Ca^{2+}-CaM phosphorylating system of increasing concentrations of Chaps were tested, phosphorylation, both of αKAP and of triadin, were found to be inhibited at lower concentrations than those inhibiting the autophosphorylation of 60-kDa CaM K II, based on autoradiographic evidence (not shown). It also should be pointed out that phosphorylation of triadin is very marked with fast-twitch muscle TC, but much less so with TC from slow-twitch muscle. Structural interactions between triadin N-terminal cytoplasmic segment and RyR1, resulting in inhibition of RyR1 at the single Ca^{2+}-channel level, have been reported (32), and there is equally emerging evidence from this laboratory for a single CaM K II phosphorylation site on triadin N-terminal end. Therefore, one might be led to speculate as to whether αKAP is a phosphorylation target of CaM K II, having a regulatory role on SR Ca^{2+}-release, somehow analogous to that exerted by PLB on SR Ca^{2+}-transport in slow-twitch muscle.

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