αKAP is an anchoring protein for a novel CaM kinase II isoform in skeletal muscle

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Ca2+/calmodulin-dependent protein kinase II (CaM kinase II) is present in a membrane-bound form that phosphorylates synapsin I on neuronal synaptic vesicles and the ryanodine receptor at skeletal muscle sarcoplasmic reticulum (SR), but it is unclear how this soluble enzyme is targeted to membranes. We demonstrate that αKAP, a non-kinase protein encoded by a gene within the gene of α-CaM kinase II, can target the CaM kinase II holoenzyme to the SR membrane. Our results indicate that αKAP (i) is anchored to the membrane via its N-terminal hydrophobic domain, (ii) can co-assemble with catalytically competent CaM kinase II isoforms and target them to the membrane regardless of their state of activation, and (iii) is co-localized and associated with rat skeletal muscle CaM kinase II in vivo. αKAP is therefore the first demonstrated anchoring protein for CaM kinase II. CaM kinase II assembled with αKAP retains normal enzymatic activity and the ability to become Ca2+-independent following autophosphorylation. A new variant of β-CaM kinase II, termed βM-CaM kinase II, is one of the predominant CaM kinase II isoforms associated with αKAP in skeletal muscle SR.

Keywords: anchoring/CaM kinase/sarcoplasmic reticulum/SH3 binding/targeting

Introduction

Protein kinases such as cAMP-dependent protein kinase (PKA), protein kinase C (PKC), and Ca2+/calmodulin-dependent protein kinase II (CaM kinase II) phosphorylate many substrate proteins in order to coordinate diverse cellular responses to extracellular signals. But how is response specificity achieved with kinases designed to recognize many substrates? The targeting hypothesis and supporting data have provided a compelling mechanism for increasing the functional specificity of multifunctional kinases by appropriate intracellular targeting (Hubbard and Cohen, 1993; Mochly-Rosen, 1995; Pawson and Scott, 1997). Kinases can be spatially positioned near their substrates at all times, or translocate to their substrates subsequent to activation in order to improve speed and specificity in response to cell stimulation. Differential expression of anchoring proteins, some of which may directly modulate kinases, can generate distinct tissue-specific properties of a given kinase. Furthermore, compartmentalization of a kinase via an anchoring protein could affect accessibility of the kinase to its second messengers, thereby specifying a preferred receptor signaling route for its activation and/or modulating the amplitude of its basal and stimulated activities.

CaM kinase II orchestrates many cellular functions in response to Ca2+-based signals, including neurotransmitter synthesis and release, membrane excitability, synaptic plasticity, cell cycle and gene expression (reviewed in Braun and Schulman, 1995). Accordingly, the kinase has a wide tissue and subcellular distribution. There is compelling evidence for subcellular compartmentalization and targeting of the kinase. For example, although the kinase is largely soluble in transfected cells, it is also tightly bound to postsynaptic densities (PSDs) in neurons (Kelley et al., 1984), and interactions between CaM kinase II and unknown PSD proteins have been detected on SDS gels (McNeill and Colbran, 1995). A reversible translocation to this compartment was found to be regulated by activation and autophosphorylation of the kinase (Strack et al., 1997; Yoshimura and Yamauchi, 1997), and the high affinity of CaM kinase II for one of its substrates in the PSD, the NMDA receptor (Omkumar et al., 1996), could potentially play a role in this targeting. However, the only CaM kinase II targeting that is understood is that of isoforms that contain a demonstrated nuclear localization signal (NLS) (Srinivasan et al., 1994; Brocke et al., 1995). This involves a translocation into the nuclear compartment, rather than an anchoring protein, and is regulated by phosphorylation of the kinase near its NLS (Heist et al., 1998). CaM kinase II is also found in a membrane-bound form, e.g. on synaptic vesicles (Benfenati et al., 1992) and skeletal muscle sarcoplasmic reticulum (SR) (Campbell and MacLennan, 1982). We have chosen to ask how the kinase is targeted to the SR, where it modulates at least three intrinsic membrane proteins: the ryanodine receptor (the Ca2+ release channel) (Witcher et al., 1991; Wang and Best, 1992; Hain et al., 1995); phospholamban (a regulator of the Ca2+ pump protein) (Wegener et al., 1989); and the Ca2+ pump protein itself (Xu et al., 1993).

In mammals, numerous alternative spliced isoforms are generated from the four closely related α, β, γ and δ CaM kinase II genes (Tobimatsu and Fujisawa, 1989; Karls et al., 1992; Mayer et al., 1993; Nghiêm et al., 1993; Edman and Schulman, 1994; Brocke et al., 1995; Uriqui and Ashcroft, 1995; Bayer et al., 1996). These isoforms have distinct but overlapping spatial and temporal expression patterns (Tobimatsu and Fujisawa, 1989; Burgin et al., 1990; Sakagami and Kondo, 1993), suggesting different specific functions. CaM kinase II isoforms contain a C-terminal domain that is responsible for the association of
et al. of CaM kinase II was found to be essential for its action serving as anchoring protein for CaM kinase II has been identified. To date, however, no molecule targeting to the SR, PSD, cytoskeleton and other subcellular compartments. Thus, insights into the cellular function and regulation of PKA and PKC via anchoring proteins has been shown to determine which isoform(s) might be responsible for the regulation of SR proteins such as the ryanodine receptor. The predominant immunoreactive protein in the microsomal fraction was a protein band detected with the β-specific antibody, although its molecular mass of ~72 kDa is larger than the previously identified β isoforms. Based on their mobility in SDS–PAGE, the CaM kinase II isoforms detected in the microsomal pellet of skeletal muscle also include γb and δb (Figure 2A). The β-immunoreactive band and γb-CaM kinase II are highly enriched in the particulate fraction, whereas δb was also present in the soluble fraction and an additional isoform, δα, was more abundant in the soluble fraction (Figure 2A). α-CaM kinase II is not expressed in muscle.

We used RT–PCR of skeletal muscle cDNA with β-CaM kinase-specific primers to determine whether the β-immunoreactive band is a β-isoform. Sequencing of the PCR products revealed a new β-isoform in skeletal muscle, termed βM-CaM kinase II, which differs from β-CaM kinase II by an insert in the variable region coding for an additional 12 kDa peptide (Figure 2B; DDBJ/EMBL/GenBank accession No. AF069731). The β-reactive band in the microsomal fraction is, in fact, the novel βM isoform, since expression of recombinant βα-CaM kinase II in COS cells produces a β-immunoreactive protein with identical mobility in SDS–PAGE to the muscle protein (Figure 2A).

αKAP is co-localized with CaM kinase II at membranes in skeletal muscle

Since homomeric recombinant βα-CaM kinase II, like other CaM kinase II isoforms, is not membrane bound (data not shown), we reasoned that the non-kinase protein αKAP, which contains a hydrophobic sequence (Bayer et al., 1996), may associate with and target soluble isoforms to the membrane. αKAP was localized exclusively in the particulate fraction (Figure 3A); the MGI serum used in a previous study (Bayer et al., 1996) cross-reacted with an unrelated but similar-sized soluble protein, leading to its incorrect designation as a soluble protein (Bayer et al., 1996; Sugai et al., 1996). The membrane association of αKAP suggested by these experiments was further validated by a sucrose flotation assay: the vast majority of the particulate αKAP banded on top of the 45% sucrose cushion (Figure 3A).
The CaM kinase II isoforms in a microsomal fraction of skeletal muscle include the novel β₄-CaM kinase II. (A) A soluble cytosolic fraction (S) and a particulate microsomal fraction (P) of rat skeletal muscle were compared with recombinant CaM kinase II isoforms (β, β₂, β₃, δ and δ₁) and a brain protein extract (br) by immunoblotting. Kinase was detected with an anti-β-CaM kinase II antibody (left panel), an anti-γ antibody which crossreacts with β isoforms (middle) and an anti-δ antibody (right). Note that δ₄-CaM kinase II (also termed δ₂) is the only known δ isoform with a molecular mass significantly higher than δ₁ but lower than δ₃, thus facilitating its identification (Mayer et al., 1993; Edman and Schulman, 1994). (B) Structure and peptide sequence of the novel β₄-CaM kinase II. The variable region of the β-CaM kinase II gene (Karls et al., 1992) is schematically represented. The conserved exons are shaded, the δM insert and regions lacked by individual isoforms are indicated. The δM insert consists of three proline rich repeats (a, b, c) which are homologous to each other and are aligned below. Two of those repeats are also present in β₃-CaM kinase II (Uriquidi and Ashcroft, 1995). Potential phosphorylation sites for CaM kinase II and PKA (P), and a putative SH3-domain binding region (SH3 binding) are marked.

Assessment of co-localization of αKAP and CaM kinase II was refined by a sucrose step gradient of a microsomal fraction of skeletal muscle using the NLS antibody is shown and the αKAP band is indicated. αKAP was also probed in fractions of a sucrose flotation assay (1, 15:45% sucrose interphase; 2, 45:55% sucrose interphase; 3, pellet). Similar results were found with two additional antibodies against other αKAP epitopes (not shown). (B) A microsomal preparation of rat skeletal muscle was fractionated on a sucrose density gradient and the fractions (F1–F5) were examined together with the cytosolic fraction (S) and the initial low speed pellets (P1 and P2) by immunoblot with antibodies to β₃-CaM kinase II and the NLS antibody to αKAP. F1–F5 are fractions collected from the interphases on top of the 26, 32, 34, 38 and 45% sucrose cushions, respectively. (C) Ponceau S staining of total protein. The Ca²⁺ pump protein (CPP) and the Ca²⁺ binding protein calsequestrin (CS) are indicated. Total protein content of fractions F1–F5 is indicated below. The composition of the fractions are: F1, mainly plasma membrane and T tubules, as indicated by the low concentration of CPP and high concentration of the Na⁺/K⁺ ATPase (immunoblot not shown); F2, light SR and T tubules based on a high CPP concentration combined with the absence of CS; F3, F4, and F5, longitudinal and terminal cisternae of the SR based on the enrichment of both CPP and CS. The nuclear envelope is mainly present in F1 and in the low speed pellets P1 and P2, according to immunostaining with an antibody against nuclear pore complex proteins (data not shown).

These results indicate that αKAP is localized primarily in the SR membrane. Little if any is present in the plasma membrane (F1), T tubules (F1) or the nuclear envelope (F1, P1 and P2). In fraction F5, where the terminal junctions of the SR are expected to be most enriched, the αKAP concentration is slightly lower than in fraction F4 (longitudinal SR) (Figure 3B). This suggests that αKAP is present in both terminal and longitudinal SR. Overall, the results clearly demonstrate an overlapping localization...
pattern of αKAP and different CaM kinase II isoforms among the membraneous fractions of skeletal muscle.

**αKAP is a CaM kinase II anchoring protein**

We hypothesized that αKAP might serve as the anchor protein directing CaM kinase II to the SR membrane. Its C-terminal association domain might coassemble with the corresponding domain of CaM kinase II isoforms while its N-terminal hydrophobic domain would directly target the heteromer to membranes (Figure 1). We therefore tested the function of these two domains of αKAP.

**αKAP is membrane-associated via its N-terminal domain.**

A sucrose flotation assay indicated that αKAP is attached to membranes (Figure 3) and its solubilization properties are consistent with a direct membrane binding (Figure 4A). αKAP was not extracted from the particulate fraction by stepwise increases in the salt concentration up to 1.2 M KCl and remained insoluble even in 6 M urea (not shown). A partial solubilization of αKAP (~50%) was obtained with detergent (2% Triton X-100). However, complete solubilization was only achieved by a combination of detergent and salt (2% Triton X-100/0.6 M KCl) (Figure 4A).

To test the hypothesis that αKAP is membrane-anchored by its N-terminal hydrophobic domain, a recombinant αKAP lacking this region (αKAPΔh) was expressed in COS cells. In contrast to αKAP, no detergent was needed to solubilize αKAPΔh (Figure 4B), indicating that the N-terminal domain is responsible for membrane association. However, under hypotonic conditions αKAPΔh was particulate, and the requirement of KCl for complete solubilization could indicate additional interactions with insoluble compounds.

**αKAP can recruit CaM kinase II into the particulate fraction.** The first evidence for an interaction of αKAP with CaM kinase II was provided by the ability of αKAP to recruit CaM kinase II into the particulate fraction (Figure 5). We expressed various isoforms of CaM kinase II (α, β, γh or δh) with either αKAP or αKAPΔh, and extracted the transfected COS cells in the presence of KCl to produce a soluble and particulate fraction. As when individually expressed, αKAP was particulate and αKAPΔh was soluble when coexpressed with the soluble kinase isoforms. However, the relative amount of CaM kinase II in the particulate fraction was significantly higher when coexpressed with wild-type αKAP than with αKAPΔh.

**CaM kinase II binds to αKAP in an immobilized activity assay**

To investigate more directly whether αKAP binds CaM kinase II, we used a modification of an immobilized kinase assay (De Koninck and Schulman, 1998). Recombinant αKAP containing a 12 amino acid hemagglutinin (HA) peptide tag at the C-terminus (αKAPtag) was cotransfected with non-tagged α-CaM kinase II, cells were extracted with KCl/Triton X-100, adsorbed to PVC microtiter plates previously coated with anti-HA tag antibody, and kinase activity associated with the immobilized αKAPtag was measured. HA-tagged α-CaM kinase II (αCaMKtag) was similarly prepared and the immobilized enzyme, which retains normal kinase activity (De Koninck and Schulman, 1998), was used as positive control. A significant amount of kinase activity was immobilized only when α-CaM kinase II was coexpressed with αKAPtag (Figure 6). If α-CaM kinase II and αKAPtag were individually expressed and then mixed, no kinase binding was obtained (Figure 6). These results provide further evidence for the ability of αKAP to bind CaM kinase II, and suggest that this interaction involves intracellular assembly as heteromultimers.

To immobilize the same CaM kinase II activity, an ~4-fold higher CaM kinase II concentration was needed in an αCaMK–αKAPtag binding reaction than in an αCaMKtag binding reaction. All the experiments were carried out in high concentrations of salt and detergent, and this finding does not allow conclusions about the stoichiometry of the CaMK–αKAP complexes. A high ratio of αKAP to kinase subunits would interfere with inter-subunit autophosphorylation so we examined autophosphorylation in immobilized αCaMK–αKAPtag. Autophosphorylation was stimulated with Ca2+-calmodulin in the presence of 250 μM ATP and the Ca2+-independent (autonomous) CaM kinase II activity generated by the autophosphorylation was assayed. Autophosphorylation

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**Fig. 4.** αKAP is membrane associated via its N-terminal domain. αKAP was detected by immunoblotting using the NLS antibody. (A) Aliquots of the high speed pellets (P) and supernatants (S) of attempts to solubilize αKAP from the particulate fraction of muscle using different treatments (KCl, 0.6 M KCl; Tx, 2% Triton X-100; KCl/Tx, 0.6 M KCl and 2% Triton X-100) were analyzed. (B) COS cells overexpressing αKAP lacking the N-terminal domain (αKAPΔh) or wild-type αKAP (αKAP) were fractionated as indicated above, either under hypotonic conditions (ht) or in the presence of 1.1 M KCl (KCl).

**Fig. 5.** αKAP recruits different isoforms of CaM kinase II to the particulate fraction. COS cells were transfected with expression vectors for different CaM kinase II isoforms (α, β, γh or δh), either together with an αKAPΔh (Δh) or an αKAP (wt) expression vector and fractionated into a particulate high speed pellet (P) and a soluble supernatant (S) in the presence of 1.1 M KCl. Aliquots were subjected to Western blot analysis. αKAP was detected by the NLS antibody, the different CaM kinase II isoforms by calmodulin overlay.
increased autonomous activity to 70% of the maximal Ca2+/calmodulin-stimulated, indistinguishable from that seen with αCaMKtag alone, indicating that autonomous kinase activity can be generated when kinase subunits are in complex with αKAP (Figure 6).

**CaM kinase II is associated with αKAP in skeletal muscle**

The association of αKAP with CaM kinase II in rat skeletal muscle was tested by immunoprecipitation (Figure 7). Fraction F4 of a sucrose step gradient (Figure 3B) was extracted with salt and detergent, and a high speed supernatant was subjected to immunoprecipitation. Most of the tested antibodies against CaM kinase II or αKAP failed to detect their antigen under conditions needed to solubilize αKAP. However, coprecipitation of αKAP was obtained with the β-CaM kinase II–specific antibody CBβ1 (Figure 7). The data strongly suggest that αKAP is co-localized and associated with βM-CaM kinase II in αKAP and CaM kinase II associate with high affinity, maintaining their interaction at high salt and detergent concentrations used in the co-immunoprecipitation and in the immobilized kinase assay. Since αKAP contains the entire association domain of βM-CaM kinase II (Lin et al., 1987; Brocke et al., 1995; Bayer et al., 1996), co-assembly with kinase subunits is likely to utilize the same interactions that are involved in self-assembly of the kinase into homo- or heteromers (Shen and Meyer, 1998). This is consistent with the finding that αKAP and CaM kinase II associate only if coexpressed and not when mixed after independent expression. There have been several reports of CaM kinase II interacting with proteins distinct from αKAP. The regulatory domain of α-CaM kinase II interacts with synaptic vesicles, although in this case the kinase is membrane-associated by an unknown mechanism and may actually serve as an anchor for synapsin I (Benfenati et al., 1992). The physical properties of the CaM kinase II association with synaptic vesicles (Benfenati et al., 1996) make it attractive to propose that this targeting might also be achieved by an αKAP-like protein. A number of studies report interaction of the kinase with the cytoskeleton including PSDs, although no specific cytoskeletal protein has been implicated in these interactions (Sahyoun et al., 1985; Saitoh and Schwartz, 1985; McNeill and Colbran, 1995). Families of specific anchoring proteins have been characterized for PKA and PKC, two other multifunctional

**Discussion**

The concept of subcellular targeting by anchoring proteins is of major importance for understanding the specificity of signal transduction. The work presented here constitutes the first description of an anchoring protein for multifunctional CaM kinase II. αKAP exhibits three properties expected of anchoring proteins. (i) It is restricted to a specific cellular compartment, it is membrane bound and probably directly inserted into SR membranes by its N-terminal hydrophobic domain (Figures 3 and 4). (ii) It binds CaM kinase II. This binding occurs within intact cells and not during extraction of transfected cells, since significant interaction was only detected after coexpression of αKAP and CaM kinase II, but not when individually

![Fig. 6. αKAP is associated with CaM kinase II as revealed by an immobilized kinase assay. Microtiter plates were coated with anti-HA tag antibody and overlaid with overexpressed α-CaM kinase II (CaMKII), a mixture of individually overexpressed HA-tagged αKAP (αKAPtag) and α-CaM kinase II, or coexpressed αKAPtag and α-CaM kinase II. The overlaid solutions contained the same soluble kinase activity bound to the plates is presented relative to the observed activity with the αKAPtag and α-CaM kinase II coexpressing extracts. Additionally, the autonomous activity after autophosphorylation of the bound kinase from this coexpressing extract was measured. The background of the assay is 12% and has been subtracted. The error bars show the standard deviation, and the standard deviation of the baseline is given by a dotted line.](image)

![Fig. 7. αKAP is binding CaM kinase II at membranes. An aliquot of the SR membrane-containing fraction F4 was extracted with salt and detergent, and a high speed supernatant (total) was subjected to immunoprecipitation with the β-CaM kinase II–specific antibody CBβ1 (prec.) or mock precipitation with PAS but without antibody (w/o Ab). Aliquots of the high speed supernatant, the precipitates and an aliquot of the CBβ1 antibody (Ab alone) were subjected to immunoblotting. Coprecipitation of αKAP was detected using the NLS antibody. The amount of precipitates examined is equivalent to six times the amount of supernatant examined. The αKAP band is indicated.](image)
protein kinases (reviewed in Hubbard and Cohen, 1993; Mochly-Rosen, 1995; Pawson and Scott, 1997). AKAPs bind the regulatory subunits of inactive PKA tetramers; after activation the catalytic subunits dissociate to the cytosol. By contrast, RACKs recruit PKC to particulate compartments only after the kinase has been activated in the cytosol. Thus, in both cases the locus of the activation and the action of the kinase is different, which may provide a biologically important delay. For CaM kinase II a reversible translocation to the PSD following activation and autophosphorylation has been described (Strack et al., 1997; Yoshimura and Yamauchi, 1997), although the molecular basis for such targeting is not known. Our data suggest that αKAP targets CaM kinase II to the SR and that both the association with the kinase and anchoring to SR occur independently of the activation state of the kinase. Anchoring to the SR membrane brings CaM kinase II near the entry site of Ca\(^2^+\) and increases its concentration near physiological substrates, such as the ryanodine receptor and phospholamban. CaM kinase II has been shown to phosphorylate the ryanodine receptor, inactivating it and thereby modulating Ca\(^2^+\) levels via a negative feedback mechanism (Wang and Best, 1992; Hain et al., 1995). A delay in this circuit would be introduced if the kinase were required to diffuse to the ryanodine receptor following activation; such a delay would be likely to alter the frequency at which Ca\(^2^+\) waves could occur and might result in significant differences in the concentration of Ca\(^2^+\) available for intracellular signaling. Anchoring of CaM kinase II by αKAP might serve not only to restrict substrate specificity in vivo but also play a role in determining the frequency at which Ca\(^2^+\) waves could occur. The endogenous SR CaM kinase II has a different effect on the ryanodine receptor than exogenously added enzyme (Hain et al., 1995), suggesting that anchoring by αKAP might limit the access of the kinase to some of the possible phosphorylation sites on the substrate.

The hydrophobic N-terminus of αKAP is responsible for its membrane association (Figure 4B). Rather than use an unrelated anchoring protein, CaM kinase II signaling has evolved to use a gene within the α-CaM kinase II gene to introduce this hydrophobic domain into a protein that retains the association or self-assembly domain of the catalytically competent isoforms. Interestingly, the genes for two other Ca\(^2^+\)/calmodulin-dependent protein kinases, CaM kinase IV and MLCK, also encode additional non-kinase products, calsperrin and KRP, respectively (Means et al., 1991; Ohmstede et al., 1991; Collinge et al., 1992). However, these proteins probably do not function as anchoring proteins. In contrast, KRP may actually disrupt the localization of MLCK by competing for myosin binding (Shirinsky et al., 1993; Silver et al., 1997).

Database searches reveal homology of the hydrophobic domain of αKAP to signal peptides, which are responsible for import into the endoplasmic reticulum (reviewed in Schatz and Dobberstein, 1996). However, unlike classical signal peptides, αKAP is not subject to posttranslational cleavage. Thus, the N-terminal domain of αKAP might represent a signal/anchor sequence combination, being responsible for both targeting to the SR and anchoring in its membrane, as described for cytochrome P-450 (Sakaguchi et al., 1987). Like αKAP, cytochrome P-450 lacks positive charges on the N-terminal side of the hydrophobic signal sequence. In fact, introduction of basic amino acids at this position converts cytochrome P-450 into a secreted protein (Szczesna-Skorupa et al., 1988). Curiously, αKAP also contains a sequence identical to the nuclear localization signal that targets α\(_{\text{h}}\)-CaM kinase II to the nucleus (Brocke et al., 1995; Bayer et al., 1996). αKAP is not targeted to the nucleus despite this sequence, perhaps because it is prevented by the dominant membrane anchoring function of the hydrophobic sequence. This distribution is more likely to occur if there is co-translational membrane insertion of αKAP rather than an initial assembly of αKAP with kinase into a holoenzyme, with a posttranslational membrane association.

An important role of anchoring proteins may be to bring different effector molecules together to form signaling complexes (reviewed in Pawson and Scott, 1997). αKAP–CaM kinase II holoenzymes may bind NLS receptors. The binding of NLS receptor to α\(_{\text{h}}\)-CaM kinase II has recently been shown to be phosphorylation dependent (Heist et al., 1998). Since an identical NLS sequence with the critical phosphorylation site is also present in αKAP, αKAP may function in a regulated sequestration of NLS receptors. Additionally, the insertion of β\(_{\text{h}}\)-CaM kinase II contains a consensus sequence for SH3-domain binding, which was also found in the pancreatic β\(_{\text{s}}\) isoform (Urizquidi and Ashcroft, 1995) and might represent a link to tyrosine kinase pathways, as described for a Ste20-related serine kinase (Anafi et al., 1997).

αKAP provides an example for a novel mechanism for achieving tissue-specific compartmentalization of effector molecules by differential use of the modular structure of proteins and genes. Examination of additional proteins interacting with αKAP and CaM kinase II may promote further insight about control of intracellular signalling by protein networks.

Materials and methods

Expression of αKAP and CaM kinase II in COS cells

All expression constructs used are derivatives of SRα and constructed as previously described (Nghiem et al., 1993; Edman and Schulman, 1994; Brocke et al., 1995; De Koninck and Schulman, 1998). For cloning of the αKAP expression vector, a PCR product was generated using a murine skeletal muscle cDNA as template and the primer combination AK32–AK2, essentially as described (Bayer et al., 1996), and directly cloned in PCR\({\text{TMM}}\) (Invitrogen, Carlsbad, CA). The insert was then subcloned into the EcoRI site of SRα, and the DNA sequence was confirmed (Bayer et al., 1996). Note that the αKAP homologue in rat (Sugai et al., 1991; Collinge et al., 1992) is identical to the murine αKAP (Bayer et al., 1996). The vector for expression of αKAPtag was generated in a similar fashion, except that HA-tag was used as 3′-primer (5′-ACAGATCTGGGGGCGCCCTTCCTGCTGCGGATTATCCTATAGCTGCGCCATGCTGCGCAGGGGGA-3′) to insert an HA epitope tag at the C-terminus of αKAP. The αKAPα expression vector was constructed using a Pol-EcoRI fragment of the PCR product with the 5′-primer K2 (5′-GCCTGCGACAAGCGCCACCATTGCGGCTGCTCCAGGAGGAAAGA-GAAGA-3′) to delete the 21 N-terminal hydrophobic amino acids. For generating a β\(_{\text{h}}\)-CaM kinase II expression construct, a SacII–ApoI fragment of a RT–PCR product from skeletal muscle with the primer combination B2-5′-ATCTACACACTATGTCGAGCAGAGTAC-3′ and B3 (Brocke et al., 1995) was inserted into the respective sites of the β-CaM kinase II expression vector.

 COS-7 cells were transfected by the CaPO\(_4\) method as described (Srinivasan et al., 1994). In cotransfections, 10 \(\mu\)g of αKAP and 3 \(\mu\)g of CaM kinase II expression vector were used for one 10 cm dish. The cells were harvested 68–76 h after transfection.
Flotation assay

The flotation assay and the sucrose step gradient followed a modification of previously described protocols (Satoh et al., 1984; Leibovitch et al., 1993) carried out at 4°C. Rat skeletal muscle (40 g) was homogenized in 160 ml of 10 mM HEPES pH 7.4, 5 mM EDTA, 1.2 mM EGTA, 10% sucrose, 1 mM PMSF, 10 μg/ml leupeptin and 10 μg/ml pepstatin with 5 × 20 s bursts in a Waring blendor. The homogenate was then subjected to two low speed centrifugations (10 min at 4000 g and 20 min at 10 500 g) and the pellets were saved (P1 and P2). The supernatant was filtered through cheesecloth, supplemented with KCl to a final concentration of 0.5 M, stirred for 30 min and centrifuged for 45 min at 186 000 g (40 000 r.p.m. in a 45Ti rotor, Beckman). The cytosolic supernatant was saved and the microsomal pellet was resuspended in 4 mM HEPES pH 7.4, 0.4 M KCl, 26% sucrose, 1 mM PMSF, 10 μg/ml leupeptin and 10 μg/ml pepstatin for further fractionation. The sucrose step gradient was composed of 4 ml 45% sucrose as bottom layer followed by 7 ml cushions of 38, 34 and 32% sucrose. Ten milliliters of the microsomes resuspended in 26% sucrose were loaded on top and overlaid with 15% sucrose. The gradient was buffered with 4 mM HEPES pH 7.4 and contained 0.4 M KCl. After 16 h centrifugation in a SW28 rotor (Beckman) at 22 000 r.p.m., the interphases between the sucrose layers were harvested, and washed by dilution and recentrifugation. The protein concentrations of the rehomogenized pellets were determined by the method of Bradford (Bradford, 1976) using bovine serum albumin (BSA) as standard.

Microsomal preparations, sucrose step gradient and flotation assay

Microsomal preparations of murine skeletal muscle were prepared for rat, however in small scale using a Viris '45' homogenizer. In this case, sucrose step gradient assay, microsomal pellets were used as bottom layer of a gradient and overlaid with a 45% and a 15% sucrose cushion.

Solubility tests

COS cells were harvested in ice-cold phosphate-buffered saline and pelleted. Cells were disrupted by sonication in homogenization buffer (HB): 50 mM PIPES pH 7.0, 1 mM EGTA, 2 mM DTT, 10% glycerol, 1 mM PMSF, 10 μg/ml leupeptin and 10 μg/ml pepstatin. Skeletal muscle and brain were homogenized using a Viris '45'. The homogenates were then subjected to a 100 000 g centrifugation (50 000 r.p.m. in a TLA100.2 rotor, Beckman) at 4°C for 1 h, and the particulate high speed pellet was resuspended in HB, restoring the original volume of the homogenate. For solubilization of αKAP or κKAPαΔh, the HB was supplemented as indicated, and the homogenate was tumbled for 30–40 min in 4°C prior to the second high speed centrifugation. When κKAP was coexpressed with CaM kinase II, 1.1 M KCl was added before the fractionation.

Immunodetection and calmodulin overlay

Equal sample volumes or, in the case of the microsomal fractions, equal protein amounts were analyzed by Western blotting with immunodetection or calmodulin overlay using the ECL system (Amersham) as described (Srinivasan et al., 1994; Brocke et al., 1995). The protein transfer was routinely examined by Ponceau S staining of the blots. Antibody binding was carried out in the presence of 2% non-fat dry milk, calmodulin binding in presence of 1% BSA. All binding and washing solutions contained 0.1% Tween-20. The following antibodies were used: polyclonal antibodies with anti-αKAP immunoreactivity NLS (gift of Dr M.Srinivasan, Department of Neurobiology, Stanford University School of Medicine, Stanford, CA), MGI (Bayer et al., 1996) and RU16 (gift from Dr A.Czernik, Laboratory of Molecular and Cellular Neuroscience, The Rockefeller University, NY) (Benfenati et al., 1992); the anti-CaM kinase II antibodies CBβ1 (anti-β; Gibco-BRL, Gaithersburg, MD), anti-γCaMKII (Santa Cruz Biotechnology Inc., Santa Cruz, CA) and δ tail (anti-δ; gift of Dr M.Srinivasan); an antibody against Na+/K+ ATPase (αNKA; gift from Dr W.J.Nelson, Department of Molecular and Cellular Physiology, Stanford University School of Medicine, Stanford, CA) (Mays et al., 1995) and an antibody against nuclear pore complex proteins (mAb 414; Babco, Richmond, CA).

Immobilized kinase assay

COS cells transfected with HA-tagged constructs were extracted with HB containing 0.1 M M KCl and 2% Triton X-100. The extracts were centrifuged and the 100 000 g supernatants were used as the source of enzyme to be immobilized in microtiter plates and assayed as described (De Koninck and Schulman, 1998), with the exception that the binding reaction was carried out in the presence of 0.6 M KCl and 2% Triton X-100. Non-specific activity was determined using extracts of non-transfected COS cells.

Immunoprecipitation

Fraction F4 of the sucrose step gradient was solubilized with Triton X-100-KCl as described above. One hundred microliters (40 μg of total protein) were incubated with the β-CaM kinase II specific antibody CBβ1 (1:330) for 1 h at 4°C on a roller, then 100 μl of 50% protein A-Sepharose (PAS) containing 2.5% BSA were added and the mixture incubated for an additional 1 h. The PAS was harvested by centrifugation at 2000 g for 1 min. The pellet was washed once in incubation buffer (HB supplemented with 2% Triton X-100, 0.6 M KCl and 2.5% BSA) and then incubated with 50 mM PIPES pH 7.0, 150 mM NaCl, 1 mM EDTA and 1% Triton X-100. The pellet was boiled for 10 min in 100 μl SDS-loading buffer, and a 15 μl aliquot was analyzed by immunoblotting.

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References


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Note added in proof

One of the PSD proteins that interacts with CaM kinase II has been shown to be the NR2B subunit of the NMDA receptor which may serve as a target for translocation of the autophosphorylated kinase to the PSD [Strack,S. and Colbran,R.J. (1998) Autophosphorylation-dependent targeting of calcium/calmodulin-dependent protein kinase II by the NR2B subunit of the N-methyl-d-aspartate receptor. J. Biol. Chem., 273, 26354–26359].