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Coordination of Three Signaling Enzymes by AKAP79, a Mammalian Scaffold Protein

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Multivalent binding proteins, such as the yeast scaffold protein Sterile-5, coordinate the location of kinases by serving as platforms for the assembly of signaling units. Similarly, in mammalian cells the cyclic adenosine 3′,5′-monophosphate–dependent protein kinase (PKA) and phosphatase 2B [calcineurin (CaN)] are complexed by an A kinase anchoring protein, AKAP79. Deletion analysis and binding studies demonstrate that a third enzyme, protein kinase C (PKC), binds AKAP79 at a site distinct from those bound by PKA or CaN. The subcellular distributions of PKC and AKAP79 were similar in neurons. Thus, AKAP79 appears to function as a scaffold protein for three multifunctional enzymes.

Control of multiple cellular events by protein phosphorylation requires many levels of regulation in order to generate specific cellular responses. One regulatory mechanism is that kinases and phosphatases are maintained at discrete cellular locations through their interaction with targeting proteins (*1*). Enzymes may be positioned in close proximity to specific substrates, which then can be efficiently modified in response to the appropriate signals. Evidence supporting this model has shown that protein tyrosine kinases and phosphatases couple to downstream cytoplasmic enzymes through adapter proteins that contain SH2 and SH3 domains (*2*). Serine-threonine kinases and phosphatases are also maintained by scaffold proteins or anchoring proteins. In yeast, the scaffold protein Sterile-5 (STE5) provides a framework to order successive members of a yeast mitogen-activated protein kinase cascade, thereby permitting sequential activation of each enzyme in the pheromone mating response (*3*). In neurons, PKA and CaN are both localized to postsynaptic densities (PSDs) by association with AKAP79, which positions both enzymes close to key neuronal substrates (*4*). Because other neuronal signaling enzymes are present at the PSD (*5*), we investigated their potential to associate with the anchoring protein AKAP79.

PKC, a family of serine-threonine kinases, is tethered to the PSD through association with binding proteins (*6*). We used a solid-phase binding assay (overlays) with PKC as a probe (*7*) on bovine brain extracts (*8*) to detect several PKC-binding proteins, including a protein that migrated with the same mobility as a prominent RII-binding protein of 75 kD (*9*). This band corresponds to AKAP75, the bovine homolog of AKAP79 (*10*), indicating that the anchoring protein could bind both RII and PKC. Indeed, recombinant AKAP79 bound to

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PKC in the presence of Ca^{2+} and phosphatidylserine (Fig. 1A). The α, βI, and βII isoforms of PKC interacted with AKAP79, whereas other signaling enzymes that are associated with the PSD, such as Ca^{2+}-calmodulin–dependent protein kinase II and the type I phosphatase, did not bind the AKAP (*11*). Phosphorylation of AKAP79 by PKC had no qualitative effect on binding (Fig. 1, A and B). This result suggests that the AKAP binds in a different manner from other PKC-substrate–binding proteins such as MARCKS (myristoylated alanine-rich C kinase substrate) and γ-adducin, for which phosphorylation regulates association with PKC (*12*). We mapped the PKC-binding region by screening a family of AKAP79 fragments (Fig. 1C) (*13*). Fragments encompassing the first 75 residues of AKAP79 bound PKC, whereas COOH-terminal fragments containing the RII- and CaN-binding regions did not (Fig. 1D). These data imply that PKC binds to AKAP79 at a site that is distinct from those bound by RII and CaN (*4*, *14*).

Because basic and hydrophobic regions are determinants for binding of certain proteins to PKC (*15*), we focused on a region located between residues 31 to 52 of AKAP79 (Fig. 2A). A peptide encompassing this region specifically blocked the interaction of AKAP79 with PKC in the overlay assay (Fig. 2B) but did not affect RII binding to the AKAP (Fig. 2C) (*16*). Conversely, the RII anchoring inhibitor peptide [AKAP79(390–411)] did not affect PKC binding (Fig. 2B) but did block interaction with RII (Fig. 2C). This result indicates that residues 31 to 52 represent the principal determinants for PKC binding. Many kinases or phosphatases bound to anchoring proteins are maintained in an inactive state (*1*, *4*). Accordingly, recombinant AKAP79 protein inhibited PKC activity (*17*) with a half-maximal inhibition (IC_{50}) of 0.35 ± 0.06 μM (n = 3) (Fig. 2D). In addition, the AKAP79 peptide (residues 31 to 52) and a recombinant AKAP79 fragment (residues 1 to 75) inhibited PKC activity with IC_{50} values of 2.0 ± 0.6 μM (n = 4) and 1.6 ± 0.3 μM (n = 4), respectively (Fig. 2D). In contrast, AKAP79(31–52) did not inhibit the activity of the catalytic subunit of PKA (Fig. 2D) (*18*). Inhibition of PKC activity by AKAP79(31–52) was mixed, with an apparent inhibition constant (K_i) of 1.41 ± 0.28 μM (n = 3) (Fig. 2E). The secondary plot of the Michaelis constant divided by the maximal velocity (K_m/V_{max}) as a function of inhibitor concentration was nonlinear, suggesting binding at more than one site (Fig. 2E, inset). Preincubation of AKAP79(31–52) with excess calmodulin prevented inhibition of PKC activity (*19*). We propose that PKC is localized and inhibited by AKAP79 in a Ca^{2+}- and phosphatidylserine-dependent manner, and additional signals, such as Ca^{2+}-calmodulin, may be required to release the enzyme.

The interaction of PKC and AKAP79 was independently confirmed by coprecipitation of the complex from human embryonic kidney (HEK) 293 cells in the presence of exogenous recombinant AKAP79 (Fig. 3A) (*20*). PKC was not coprecipitated with AKAP79 in the absence of exogenous AKAP79 probably because of low endogenous amounts of AKAP79 in the lysates (Fig. 3A). Similar results were obtained when AKAP79 was added to bovine brain extracts or incubated with purified PKC-βII (*21*) in vitro in the presence of Ca^{2+} and

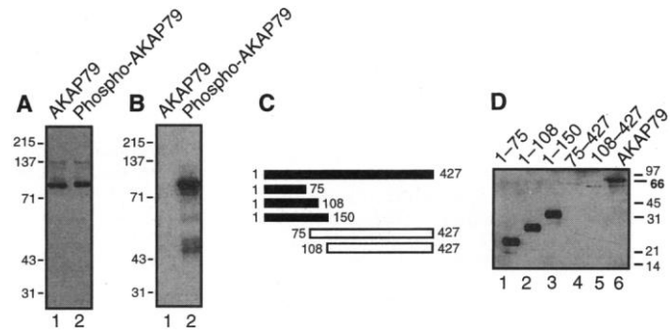

Fig. 1. Binding of PKC to AKAP79. (**A**) PKC binds nonphosphorylated and phosphorylated AKAP79 (lanes 1 and 2, respectively). Purified recombinant His-Tag AKAP79 (2 μg) was phosphorylated by partially purified PKC (100 ng) in a reaction mixture containing 50 mM tris-HCl (pH 7.4) phosphatidylserine (100 μg/ml), 1 mM EGTA, 1.2 mM calcium, 5 mM magnesium chloride, and 1 mM DTT in the absence (lane 1) or presence (lane 2) of 25 μM [γ-^{32}P]ATP for 90 min at 30°C. Samples were separated on a 10% SDS–polyacrylamide gel electrophoresis (PAGE) gel, transferred to nitrocellulose, and assayed for PKC binding by overlay analysis (*7*). (**B**) Autoradiograph showing PKC phosphorylation of AKAP79. (**C**) Schematic diagram showing the location of the AKAP79 deletion mutants (*13*). (**D**) Purified fragments (2 μg of 1–75, 1–108, and 1–150 AKAP79 and 1 μg of 75–427, 108–427, and full-length AKAP79) were separated by SDS-PAGE on a 15% gel, transferred to nitrocellulose, and assayed for binding by PKC overlay. Molecular size markers are indicated on the right in kilodaltons.

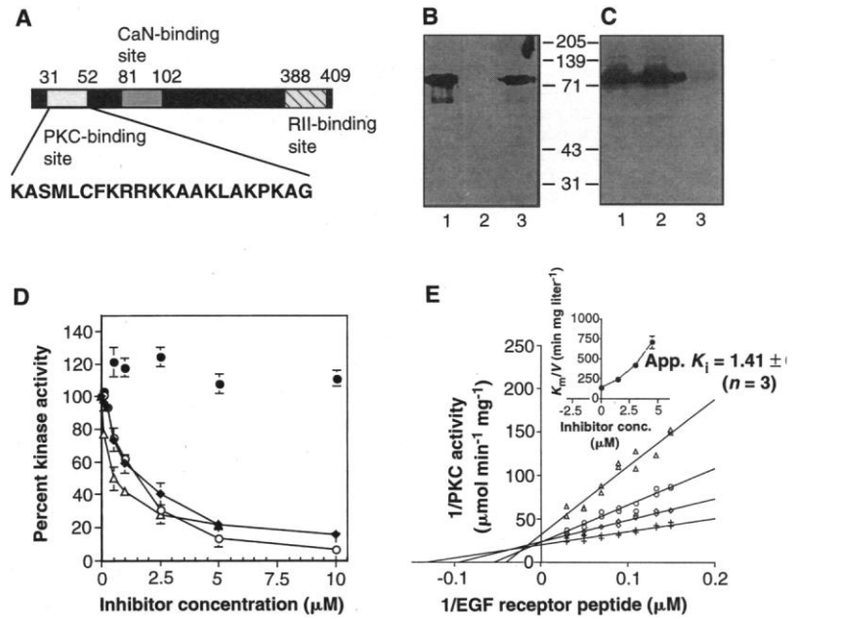

Fig. 2. Inhibition of PKC activity by AKAP79. (**A**) Schematic representation of AKAP79 showing putative binding sites for PKC, CaN, and RII. The amino acid sequence for residues 31 to 52 is indicated (*29*). (**B**) Recombinant AKAP79 was blotted and PKC overlays (*7*) were done in the absence (lane 1) and presence of either 1.5 μM AKAP79(31–52) (lane 2) or 1.5 μM RII-anchoring inhibitor peptide AKAP79(390–412) (lane 3) with ~12.5 nM PKC. (**C**) ^{32}P-RII (100 cpm/μl) overlays (*16*) were done under the same conditions as in (B). (**D**) Dose-response curve of PKC activity in the presence of recombinant AKAP79 (△), AKAP79(31–52) (◆), and residues 1 to 75 of recombinant AKAP79 (○). AKAP79(31–52) did not inhibit PKA activity (●). Values shown are the mean ± SEM (n ≥ 3). (**E**) Lineweaver-Burk plot of PKC phosphorylation in the absence of inhibitor peptide (+) and in the presence of 1.5 μM (◇), 3 μM (○), and 4.5 μM (△) AKAP79(31–52) peptide. Inset shows the secondary plot of K_m/V_{max} as a function of AKAP79(31–52) concentration and the apparent K_i value. Values shown are the mean ± SEM (n = 3). PKC and PKA were assayed as described (*17*, *18*).

phospholipid (*19*). Further evidence for the association of PKC and AKAP79 was provided by double-immunofluorescence staining (*22*) of HEK 293 cells overexpressing AKAP79 (*23*). PKC and AKAP79 exhibited limited staining at the cell periphery in mock-transfected cells (Fig. 3B), but the staining pattern was markedly enhanced in transfected cells, demonstrating that overexpression of AKAP79 increases PKC targeting to the periphery of the cell (Fig. 3B). Collectively, our data indicate that PKC binds AKAP79, and it is likely that this complex occurs in vivo.

AKAP79 may act as a signaling scaffold for the subcellular targeting of PKA, CaN, and PKC. To demonstrate that these enzymes bind to AKAP79 in mammalian cells, we isolated ternary complexes from bovine brain. We used affinity purification of the R subunit of PKA on adenosine 3′,5′-monophosphate (cAMP)–agarose to copurify AKAP75 (Fig. 4A) (*24*). If PKC were bound to the complex via the AKAP, then we would expect PKC to be present after elution of bound proteins with cAMP. Immunoblotting analysis demonstrated that PKC was indeed present (Fig. 4B), showing that RII and PKC can bind simultaneously to AKAP75. In separate experiments, a ternary complex of CaN, PKC, and AKAP75 was isolated from bovine brain extracts by immunoprecipitation with antibodies against CaN A subunit (*25*). Immunoblotting demonstrated that the precipitated complex contained PKC (Fig. 4C), whereas control experiments with nonimmune serum were negative. This result indicated that CaN and PKC can bind to the AKAP simultaneously; therefore, different combinations of PKA, CaN, and PKC can bind the AKAP in bovine brain. Isolation of a quaternary complex was not feasible because of the low stoichiometry of binding for each enzyme to the AKAP (*4*, *26*) and the probable transient nature of the protein-protein interactions.

AKAP79 appears to be a mammalian scaffold protein that coordinates the location and activity of three prominent signaling enzymes. Although AKAP79 functionally resembles the yeast scaffold protein STE5, there are differences between the two signaling scaffolds. The AKAP79 complex is likely to respond to three distinct activation signals, whereas a single upstream event, the activation of STE20, is sufficient to transduce signals from one kinase to the next in the STE5 scaffold (*3*). In addition, the AKAP signaling complex is targeted to sites near the postsynaptic membrane in neurons. Immunoflourescence studies demonstrate that PKC and the AKAP are concentrated at sites below the plasma membrane at the cell body and in dendritic bundles of hippocampal neurons (Fig. 4D). The dendritic staining pattern is consistent with the colocalization of both proteins at the PSD. Targeting to the PSD would ensure that each enzyme in the signaling complex is well placed to receive signals transduced across the synapse and that each enzyme is colocalized with specific substrate proteins. Potential substrates include α-amino-3-hydroxy-5-methyl-4-isoxazoleproprionic acid (AMPA)–kainate and Ca^{2+} channels, which are modulated by anchored PKA, and *N*-methyl-D-aspartate (NMDA) receptors, which are activated by PKC and attenuated by CaN (*27*). PKC may also modulate the targeting of certain NMDA receptor subtypes to the postsynaptic membranes (*28*). Thus synaptic signaling events appear to be coordinated, and we propose that this occurs through a modular scaffold protein, AKAP79, that anchors two second messenger–regulated kinases and one phosphatase.

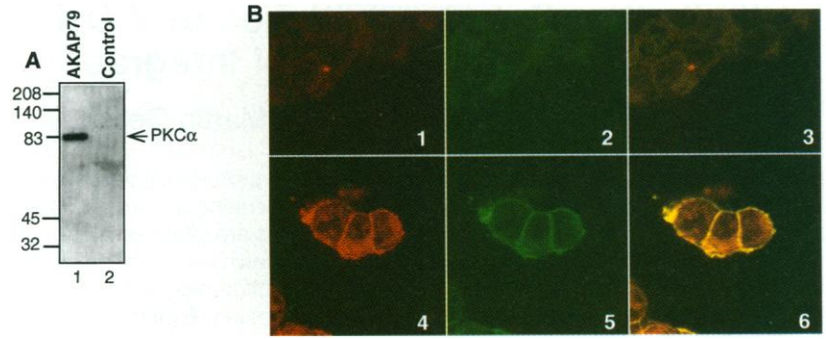

Fig. 3. Cellular association and colocalization of PKC and AKAP79. (**A**) Coprecipitation of AKAP79 and PKC from HEK 293 cell lysates (*20*). Lysates (1.2 mg) were incubated in the presence (lane 1) or absence (lane 2) of exogenous recombinant AKAP79 (2 μg) and immunoprecipitated with a polyclonal antibody to AKAP79 (2503). Precipitates were blotted and probed with a monoclonal antibody to PKCα (M4) (*7*). (**B**) Stable HEK 293 cell lines expressing pcDNA3 (upper panels) and AKAP79 (lower panels) were stained with antibodies to PKC (M4) (panels 1 and 4) or AKAP79 (2503) (panels 2 and 5) and analyzed by confocal microscopy (*22*). Double staining for PKC and AKAP79 is displayed by superimposing the images (panels 3 and 6).

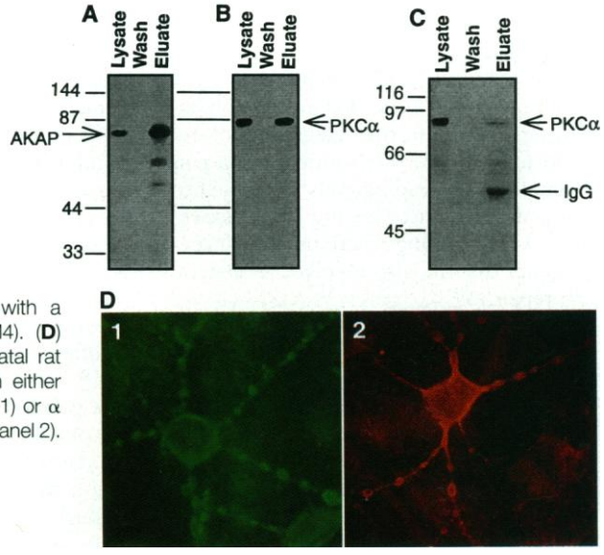

Fig. 4. The AKAP79 signaling scaffold. (**A** and **B**) The R subunit of PKA was purified from bovine brain by affinity chromatography with cAMP-agarose (*24*). Protein blots were probed with ^{32}P-RIIα to detect AKAPs (A) or with a monoclonal antibody to PKCα (M4) (B). (**C**) CaN from bovine brain extracts was immunoprecipitated with affinity-purified antibodies to the CaN A subunit (*25*). Protein blots were probed with a monoclonal antibody to PKCα (M4). (**D**) Confocal microscopy (*22*) of neonatal rat hippocampal neurons stained with either antibody to AKAP79 (2503) (panel 1) or α and β PKC-specific antibody (M7) (panel 2).

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20. HEK 293 cells were collected in hypotonic buffer containing protease inhibitors and sonicated three times for 5 s. Total lysates (1.2 mg in 300 μl) were incubated overnight in the absence or presence of 2 μg of purified recombinant AKAP79. Samples were incubated with 4 μg of affinity-purified polyclonal antibody to AKAP79 (2503) and precipitated with 40 μl of a 50% solution of protein A–Sepharose (Sigma). Complexes were washed twice in hypotonic buffer, twice in low-salt buffer [50 mM Hepes (pH 7.4), 0.15 M NaCl, 0.1% NP-40, 1 mM EDTA, and 0.1% SDS], twice in high-salt buffer (50 mM Hepes, 0.5 M NaCl, 0.1% NP-40), and twice in low-salt buffer. The precipitated complexes were boiled for 5 min in 20 μl of SDS–sample preparation buffer and immunoblotted.
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23. HEK 293 cells were transfected with Lipofectin Reagent (Life Technologies) with either pcDNA3 or pcDNA3 containing a 1312–base pair Hind III–Not I fragment encoding AKAP79. After 24 hours, G418 (0.5 mg/ml; Sigma) was added to select for stable cells overexpressing AKAP79. Cells were maintained in media containing G418 (0.2 mg/ml). Protein immunoblot analysis demonstrated increased expression of AKAP79, whereas PKCα expression remained constant.
24. Regulatory subunits of PKA were purified with the use of cAMP-agarose as described [R. L. Potter, P. H. Stafford, S. S. Taylor, *Arch. Biochem. Biophys.* **190**, 174 (1978)] with modifications (*4*). The complex from bovine brain lysates (*8*) was eluted from cAMP-agarose with 75 mM cAMP.
25. Immunoprecipitation of CaN was achieved by incubating bovine brain lysates (1 mg protein) (*8*) with affinity-purified antibodies to the CaN A subunit (8 μg) for 4 hours at 4°C. Proteins were precipitated as described (*20*).
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29. Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; F, Phe; G, Gly; K, Lys; L, Leu; M, Met; P, Pro; R, Arg; S, Ser; T, Thr; and V, Val.
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Similarities Between Initiation of V(D)J Recombination and Retroviral Integration

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In the first step of V(D)J recombination, the RAG1 and RAG2 proteins cleave DNA between a signal sequence and the adjacent coding sequence, generating a blunt signal end and a coding end with a closed hairpin structure. These hairpins are intermediates leading to the formation of assembled antigen receptor genes. It is shown here that the hairpins are formed by a chemical mechanism of direct trans-esterification, very similar to the early steps of transpositional recombination and retroviral integration. A minor variation in the reaction is sufficient to divert the process from transposition to hairpin formation.

Functional immunoglobulin and T cell receptor genes are assembled during vertebrate lymphoid development from separate gene segments. This DNA rearrangement, called V(D)J recombination, takes place at recombination signal sequences (RSSs) that specify the border of the coding segments (*1*). Double strand breaks (DSBs) at the RSS border depend on expression of the *RAG1* and *RAG2* genes (*2*) and are probably intermediates in this recombination reaction (*3*, *4*). After DSB formation, a pair of signal ends or coding ends is coupled to form a signal joint or coding joint, respectively. These joining reactions require several genes that are also involved in general DSB repair (*4*).

We recently developed a cell-free assay in which specific cleavage at RSSs requires only the RAG1 and RAG2 proteins (*5*, *6*). An oligonucleotide containing one RSS serves as a substrate and is cut efficiently. A DNA species containing a nick in the top strand (as drawn in Fig. 1) at the border of the RSS and the flanking ("coding") sequence was found to be the precursor to a DSB. The products of the cleavage reaction are a blunt, 5′-phosphorylated signal end and a coding end with a hairpin structure. These are the same species detected in recombinationally active cells.

Formation of the new phosphodiester bond at the tip of the hairpin requires energy. Because no adenosine triphosphate (ATP) or other high-energy cofactor is present in the cleavage reaction, the energy is likely to be derived from one of the broken phosphodiester bonds. The energy of the bond broken in the initial nicking reaction does not appear to be conserved: DNA substrates with a preexisting nick at this position are efficiently converted into hairpins (*6*). Thus, the energy of the phosphodiester bond in the bottom strand opposite the nick must be conserved, either through a covalent protein-DNA intermediate or by direct trans-esterification. A

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