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# AKAP MEDIATED SIGNAL TRANSDUCTION

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**Key Words** PKA, A-kinase anchoring protein, multivalent signaling platforms, cAMP, compartmentalization

■ **Abstract** Compartmentalization of cyclic AMP-dependent protein kinase (PKA) is achieved through association with A-kinase anchoring proteins (AKAPs). AKAPs are a group of structurally diverse proteins with the common function of binding to the regulatory subunit of PKA and confining the holoenzyme to discrete locations within the cell. This mode of regulation ensures that PKA is exposed to isolated cAMP gradients, which allows for efficient catalytic activation and accurate substrate selection. Several AKAPs coordinate multiple members of signaling cascades, effectively assembling upstream activators and downstream effectors within the same macromolecular complex. Consequently, AKAPs may serve as points of integration for numerous signaling pathways. This review details the most recent advances in our understanding of the various biological functions dependent upon AKAP-anchored signaling complexes.

## INTRODUCTION

Extracellular signals such as hormones, neurotransmitters, and growth factors regulate a wide variety of cellular activities including ion channel modulation, neuronal excitation, cell growth, and cell differentiation (1–3). Intracellular transduction systems receive these signals via receptors and transmit them quickly and precisely, which results in the amplification of specific biological responses. Since cells often are exposed to several messengers simultaneously, maintaining the fidelity of these networks is crucial to elicit appropriate physiological responses. Phosphorylation represents a major reversible mechanism for activation and deactivation of components in a signaling pathway. Given that many kinases and phosphatases have broad substrate specificities, a fundamental question is how they accurately select effector molecules. A principal strategy employed to achieve this specificity is compartmentalization of signaling enzymes (4). This review highlights the most recent advances in our understanding of signal transduction events mediated by A-kinase anchoring protein (AKAP) compartmentalized signaling complexes.

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## Cyclic AMP-Dependent Protein Kinase

Activation of cyclic AMP-dependent protein kinase (PKA) is one of the best-characterized signaling pathways. PKA is a serine/threonine kinase composed of two catalytic (C) subunits held in an inactive state by association with a regulatory (R) subunit dimer (5–9). The catalytic subunits are encoded by three different genes, C$\alpha$, C$\beta$, and C$\gamma$, while the regulatory subunits are expressed from four different genes, RI$\alpha$, RI$\beta$, RII$\alpha$, and RII$\beta$ (10–13). The regulatory subunit is a modular polypeptide containing an N-terminal dimerization domain, an autophosphorylation site that serves as a principal contact site for the catalytic subunit, and two cyclic AMP (cAMP) binding sites. Activation of PKA is solely executed by the major, diffusible secondary messenger cAMP (7, 14, 15). Binding of two cAMP molecules to each regulatory subunit relieves the PKA autoinhibitory contact, allows the catalytic subunits to dissociate (3, 16, 17), and results in phosphorylation of local substrates.

Two forms of the heterotetrameric PKA holoenzyme exist: type I (RI$\alpha$ and RI$\beta$ dimers) and type II (RII$\alpha$ and RII$\beta$ dimers). Type I PKA is predominantly cytoplasmic and more sensitive to cAMP than type II (7, 18). In contrast, almost 75% of type II PKA associates with specific cellular structures and organelles (reviewed in 2, 3). These differences in cAMP affinity and subcellular localization between the two PKA types are proposed to contribute to specificity in physiological responses.

## Regulation of PKA by A-Kinase Anchoring Proteins

Discrete localization of type II PKA within the cell is due chiefly to association of the regulatory subunit with nonenzymatic scaffolding proteins called A-kinase anchoring proteins (AKAPs) (reviewed in 19–22). The first AKAPs, MAP2 and AKAP75, were originally discovered as contaminants of type II PKA holoenzyme preparations (23, 24). The family has since grown to include over 50 members.

AKAPs are structurally diverse but functionally similar proteins defined on the basis of their ability to bind to PKA and coprecipitate catalytic activity (Figure 1). All contain an amphipathic helix of 14 to 18 residues that functions to bind to the N-termini of the PKA-RII dimer (25–27). Each AKAP also contains a unique subcellular targeting domain that restricts its location within the cell (20, 21). AKAPs tether inactive PKA holoenzymes at defined locations within the cell where they are poised to phosphorylate local substrate in response to cAMP activation. This method of regulation ensures that PKA is exposed to compartmentalized pools of cAMP locally generated by adenylate cyclases and phosphodiesterases, allowing for efficient catalytic activation and appropriate substrate selection (28). The final and most recently appreciated property of several AKAPs is their ability to coordinate other signaling enzymes into multivalent signaling complexes.

Generally, AKAPs predominantly anchor RII-containing PKA holoenzymes. This interaction occurs with nanomolar affinity (29) and seems to be favored in vivo over the AKAP-RI association that occurs at 100-fold lower affinity (30). However, there are a few examples where AKAP interaction is not exclusive to the

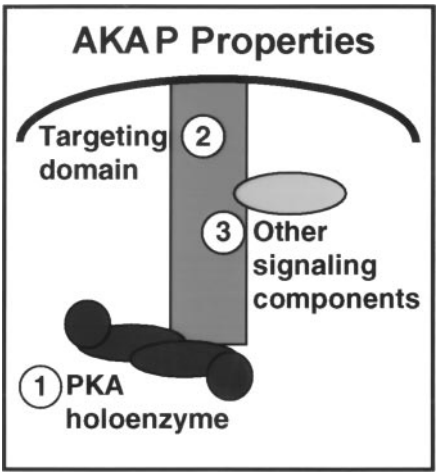


**Figure 1** Functional motifs of AKAPs. (*1*) A conserved binding domain interacts with the AKAP binding surface on the regulatory subunit dimer of PKA. (*2*) Unique targeting domains direct AKAP signaling complexes to distinct intracellular locations. (*3*) Additional binding sites for other signaling components such as kinases, phosphatases, or potential substrates.

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RII subunit. For instance, $AKAP_{CE}$ was demonstrated to interact specifically with the RI-like subunit of PKA in *Caenorhabditis elegans* (31). Furthermore, in vitro biochemical evidence suggested the existence of a family of dual function AKAPs that bind to both RI and RII (32). In fact, AKAP220 immunoprecipitates from human testis lysate contained both PKA RI and RII subunits (33), which confirms the ability of an AKAP to interact with both types of regulatory subunits in vivo.

## The Evolution of AKAPs

In yeast the PKA regulatory subunit is encoded by one gene, *BCY1*, and its subcellular localization is regulated by the available carbon source (34). Yeast grown on glucose exhibit nuclear localization of Bcy1, and carbon source-derepressed cells show an even distribution between the nucleus and cytoplasm. While Bcy1 forms a dimer structurally similar to mammalian RII (35), there is no evidence that structural homologues of AKAPs exist in yeast (21). Alternatively, one study suggests that the molecular mechanism of Bcy1 subcellular localization may be dependent upon the phosphorylation states of two serine rich clusters found in its N-terminus (36). Unphosphorylated Bcy1 was found exclusively in the nucleus, while Yak1 kinase-dependent phosphorylation of either serine cluster directed Bcy1 to the cytoplasm. A yeast two-hybrid screen for Bcy1 interactors identified Zds2 and led to the identification of Zds1 based on homology (36). Binding of Zds1 to the second cluster of phosphorylated serines in Bcy1 resulted in its cytoplasmic

retention. This localization was abolished in a *zds1* mutant strain, though Bcy1 remained phosphorylated. This suggests that phosphorylated Bcy1 binds to Zds1 and is targeted to the cytoplasm, while unphosphorylated Bcy1 remains nuclear. Additionally, there is evidence for cytoplasmic retention of Bcy1 by the first cluster of serines independent of Zds1 (36). This may suggest the existence of a different binding partner available for Bcy1 cytoplasmic retention. Thus, functional but not structural homologues of AKAPs may exist in yeast (36).

Different mechanisms for regulating the localization of the PKA holoenzyme may have evolved to handle the complexity of signaling in mammalian cells. For instance, overexpression of the unique mAKAP-targeting fragment in cardiomyocytes displaced endogenous mAKAP from its normal perinuclear location in differentiated cells (37). This evidence suggests that mAKAP may bind to a saturable targeting partner at the nuclear membrane. Consequently, the mammalian system may have developed the use of AKAPs as an additional level of specificity, while the yeast system may directly utilize PKA targeting partners without the AKAP intermediate.

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## Coordination of Multivalent Signaling Complexes by AKAPs

The most important duty of several AKAPs to date is to coordinate signal transduction complexes by recruiting multiple signaling enzymes near potential substrates. This mechanism effectively joins upstream activators with specific downstream targets within a discrete signaling complex (38). AKAP79 (39), yotiao (40), AKAP220 (41), gravin (42), WAVE (43), and mAKAP (44, 45) function in this capacity. Strikingly, there is evidence that Bcy1-interacting proteins, Zds1 and Zds2, also interact with signaling enzymes such as protein kinases Snf1 and Pkc1 (46, 47). This provides for the possibility of multivalent signaling platforms in yeast (36). Understanding these compartmentalized signal transduction units may allow us to determine whether molecular aberrations disrupting these complexes can be linked to the progression of various disease states. Two very recent reviews detail the roles of AKAPs in the coordination of multivalent signaling complexes including AKAP79 (48) and those AKAPs involved in cytoskeletal reorganization events (49). Therefore, this review focuses only on mAKAP, AKAP220, and AKAP350/AKAP450/CG-NAP/yotiao in the context of coordinating distinct signaling complexes to mediate different biological functions.

## MUSCLE AKAP (mAKAP)

Coordination of a multivalent signaling complex by muscle AKAP (mAKAP) has been described for two subcellular regions of cardiomyocytes: the perinuclear membrane and the sarcoplasmic reticulum (SR). mAKAP was originally cloned and characterized as AKAP100 (50). It was identified from a human hippocampal cDNA expression library using radioactively labeled RII$\alpha$ in a modified Western blot procedure called an RII overlay (51). Later, AKAP100 was shown to be a truncated clone, and the human and rat genes were cloned in their entirety by the

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5' RACE procedure (37). Northern blot analysis revealed a single 8-kb mRNA transcript in humans with highest levels of expression found in heart and skeletal muscle and lowest levels in lung and brain (50). The rat and human proteins contain over 2300 amino acid residues with 79% identity and are predominantly expressed in heart, brain, and skeletal muscle (37).

The RII binding domain of mAKAP maps to amino acid residues 2055 through 2072, and proline substitution into the amphipathic helix disrupts the RII/mAKAP interaction in vivo (37). Endogenous mAKAP immunoprecipitated from L6 rhabdomyosarcoma cells copurified PKI-sensitive kinase activity, which demonstrates an in vivo interaction between mAKAP and PKA (37).

The targeting domain of mAKAP consists of three spectrin-like repeat sequences (37). Subcellular localization experiments performed by transfection of GFP-tagged mAKAP or by indirect immunofluorescence localized mAKAP to the perinuclear region (37, 50) and the sarcoplasmic reticulum of cardiomyocytes (44, 52) and at intercalated discs in adult rat heart tissue sections (52). Overexpression of a spectrin-targeting fragment displaced endogenous mAKAP from its normal perinuclear location in differentiated cardiomyocytes (37). Thus, delocalization of the endogenous anchoring protein from targeting sites inside cells implies the feasibility of developing reagents specific for displacing mAKAP. This is in contrast to the Ht31 peptide reagent that globally delocalizes PKA from all AKAPs. Ht31 peptide is a fragment consisting of an amphipathic helix that binds to PKA and prevents its proper subcellular localization within the cell (29, 53, 54). Identifying AKAP-interacting proteins involved in subcellular localization would allow for the development of reagents that specifically disrupt the location of a particular AKAP. Thus, this would permit the specific dissection of each AKAP's function in a physiological process.

## mAKAP Signaling Complex at the Perinuclear Membrane of Cardiomyocytes

Functional mAKAP is implicated in the proper development of cardiomyocytes (37). In rat neonatal ventriculocytes (RNV) stimulated to adopt a differentiated hypertrophic phenotype, mAKAP expression was induced and localized to the nuclear membrane (37). It is not understood exactly which signals stimulate mAKAP expression, but it is clear that it coincides with cellular differentiation. The induction of mAKAP also caused a redistribution of PKA RII to the nuclear membranes of cardiomyocytes. Unstimulated RNVs that did not undergo differentiation contained undetectable levels of mAKAP, and RII was found in the cytoplasm (37). Thus, it appears that one of mAKAP's chief roles is to redistribute PKA to the nuclear membrane of differentiated myocytes.

In addition to this function, mAKAP coordinates a signaling complex at the perinuclear region that contains the PDE4D3 phosphodiesterase and PKA (45). Induction of mAKAP promotes the recruitment of PDE4 to the perinuclear membranes much like PKA. This localization is consistent with subcellular fractionation

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data whereby PDE4D subtypes were found in the outer perinuclear membranes of isolated cardiac nuclei (55). In vitro binding assays using bacterial purified proteins verified that PDE4D3 binds directly to an mAKAP fragment containing residues 1286 through 1831 (45). Thus, mAKAP establishes a platform for signaling at the perinuclear membrane of cardiomyocytes by directly tethering together a kinase and a phosphodiesterase.

These and other functional studies suggest that mAKAP provides the molecular framework for the assembly of a PKA/PDE negative feedback loop (45) (Figure 2). PKA phosphorylates Ser-13 and Ser-54 of PDE4D3 (56, 56a, 56b), which results in increased PDE4D3 activity (56, 56c). Consequently, exposure to cAMP results in the activation of both PDE4D3 and PKA. In cells cotransfected with mAKAP, phosphorylation of transfected PDE4D3 markedly increased as determined by antiphosphoserine blotting (45). This confirms that the mAKAP-associated PDE4D3 is a PKA substrate. Further enzymatic assays demonstrated that stimulation of mAKAP-anchored pools of PKA from rat heart extract enhanced cAMP degradation two-fold. This increase was abolished in the presence of PKA inhibitor PKI and anchoring inhibitor peptide Ht31 (45). Thus, PKA activation increases PDE4D3 activity. Finally, mAKAP-anchored PKA activity decreased upon cotransfection of PDE4D3, an effect that was blocked by the presence of PDE4 specific inhibitor rolipram (45). Thus, the model put forth by Dodge et al. (45) is that local generation of cAMP activates mAKAP-anchored PKA. PKA phosphorylates mAKAP-anchored PDE4D3, which results in increased phosphodiesterase activity. This causes increased cAMP metabolism that, in turn, attenuates PKA activity. This system demonstrates a tightly coupled cAMP signaling module mediated by mAKAP that may be significant for feedback inhibition of PKA phosphorylation events (45).

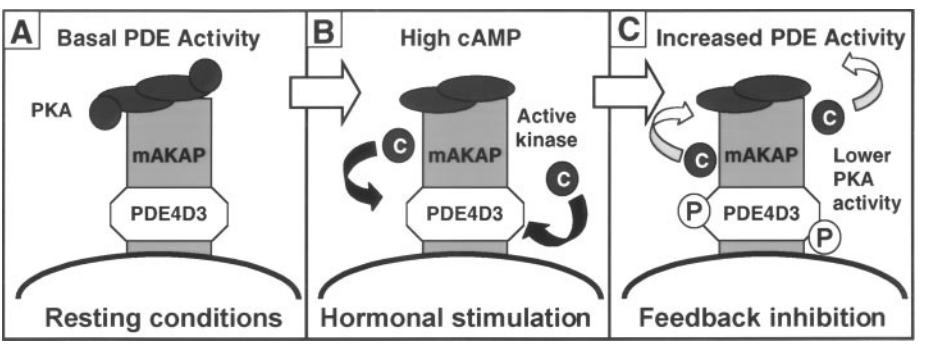


**Figure 2** Negative feedback loop coordinated by mAKAP. (*A*) Under basal conditions, PKA is inactive and the PDE maintains low intracellular concentrations of cAMP. (*B*) Upon hormonal stimulation, the generation of cAMP increases and overcomes the basal rate of PDE-mediated cAMP degradation, allowing for activation of PKA and phosphorylation of local substrates. (*C*) PKA phosphorylation of mAKAP-anchored PDE enhances PDE activity, causes increased cAMP degradation, and results in decreased PKA activity.

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The physiological consequences of the redistribution of PKA and PDE4D3 to the nuclear membrane by mAKAP are unknown; however, there are two obvious implications at this point. First, PKA phosphorylation induces certain cAMP-responsive genes that propagate the hypertrophic phenotype (57). As mentioned, expression of mAKAP is induced only upon differentiation of cells into cardiomyocytes. Thus, the redistribution may be an early response required for the full execution of differentiation and for the hypertrophic response.

Second, the chromosomal locus of the human mAKAP gene colocalizes with a genetic locus for a familial cardiomyopathy called arrhythmogenic right ventricular dysplasia (ARVD) (58). ARVD is a dominantly inherited progressive disease of the myocardium, which has an estimated incidence as high as 6 in 10,000 individuals (59). Pathologically, this disease is characterized as replacement of right ventricular myocardium with fibrofatty tissue, and it is considered to be a significant cause of ventricular arrhythmia and sudden death in adolescents and young adults (60). ARVD has been linked to mutations at one of several genetic loci (59). One locus found on chromosome 14q between markers D14S62 and D14S69 (58) coincides with the mAKAP gene location, which implicates mAKAP as a candidate gene for ARVD disease (37). Proper localization of PKA/PDE signaling complexes by mAKAP may be required to prevent the diseased state. Hence, the mAKAP complex may be important not only in heart development but also in maintenance of healthy heart function.

## mAKAP Signaling Complex at the Sarcoplasmic Reticulum

The ryanodine receptor (RyR2) is a calcium release channel found on the sarcoplasmic reticulum (SR) of cardiac muscle. Coupled gating of these channels causes the release of intracellular stores of calcium from the SR. This is thought to be important for cardiac muscle excitation-contraction (EC) coupling that controls muscle contraction (61). Cosedimentation and coimmunoprecipitation experiments determined that a complex containing mAKAP, PKA, FKBP12.6, phosphatase PP1, and phosphatase PP2A associates with the ryanodine receptor (44). Discovery of such a rich signal transduction complex associated with RyR2 potentially provides considerable molecular insight into the regulation of its function and the resulting physiological response.

Initial experiments (44) demonstrated that the ryanodine receptor was a substrate of PKA in vitro, phosphorylating GST-RyR2 on serine 2809. This kinase activity was specific for PKA, as the PKA inhibitor PKI abolished the majority of the observed phosphorylation. The molecular mechanism behind regulation of RyR2 by PKA phosphorylation involves FKBP12.6 (44), a *cis-trans* peptidyl-prolyl isomerase and cytosolic receptor for immunosupressant drugs (62, 63). It is hypothesized that one molecule of FKBP12.6 per each subunit of the tetrameric RyR2 helps keep the channel closed when cytosolic $Ca^{2+}$ is low (64, 65). Kinase assays performed on RyR2 immunoprecipitates demonstrated that PKA phosphorylation of RyR2 inhibits FKBP12.6 binding (44). The physiological consequences of

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RyR2 hyperphosphorylation manifested as altered channel function—specifically, showing increased calcium sensitivity and elevated channel activity. Thus, PKA phosphorylation of RyR2 appears to facilitate coupled gating of RyR2 channels by regulating the binding of FKBP12.6 to the channel (44).

In vivo evidence generated by back-phosphorylation and antiphosphoserine immunoblot studies demonstrated that RyR2 channels in failing hearts were hyperphosphorylated by PKA (44). These studies suggested that three out of four PKA sites on RyR2 were phosphorylated in failing hearts, whereas only one site in normal hearts was phosphorylated. This was independent of an increase in levels of PKA protein associated with RyR2. Conversely, decreased phosphatase activity could account for hyperphosphorylation since PP1 and PP2A interact with this complex (44). Pulldown experiments using GST-RyR2 fragments localized the PP1 binding domains to amino acid residues 513 through 808 and the PP2A binding domain to amino acids 1451 through 1768, though neither may be a direct interaction (44). Levels of PP1 and PP2A protein associated with RyR2 immunoprecipitates were significantly decreased in failing hearts (44), while the overall cellular PP1 protein level increases in failing hearts (66). This suggests that PKA hyperphosphorylation may be due to a local decrease in phosphatases associated with the RyR2 complex (44). Hence, there is significant evidence that regulation of RyR2 by mAKAP-anchored PKA activity may mediate SR calcium release and that deregulation of these channels in failing hearts may contribute to cardiac malfunction.

Alternatively, another study suggested that delocalized PKA contributes to heart failure (67). This study utilized the Ht31 reagent, which is an AKAP fragment consisting of an amphipathic helix that competes for PKA binding (29, 53, 54). Ht31 overlay experiments and surface plasmon resonance spectroscopy demonstrated that autophosphorylation of RII facilitated Ht31 peptide binding. This suggests that the AKAP/PKA interaction is regulated by RII autophosphorylation: an interesting observation since it is reminiscent of the subcellular localization strategy utilized by the yeast system (36). Furthermore, backphosphorylation experiments demonstrated that autophosphorylated RII was decreased in failing hearts (67). This suggests that there is less AKAP/RII interaction in failing hearts, which leads to delocalized PKA activity and a decrease in PKA substrate phosphorylation. Consequently, several mechanisms may contribute to a variety of different heart failures.

## AKAP220

AKAP220 was first identified from a rat pituitary cDNA expression library, using radiolabelled RII$\alpha$ in an overlay procedure (68). Northern analysis detected 9.7-kb and 7.3-kb transcripts in heart, lung, testis, and kidney, and the brain expressed yet a third 5.5-kb message. The highest mRNA levels were detected in brain and testis. Human AKAP220 was similarly identified by RII overlay and cloned in its entirety by 5' RACE (33). Similarly sized human mRNA transcripts were also present at high levels in human testis, brain, and heart. Rat (1129 amino acids) and

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human (1901 amino acids) AKAP220 are highly homologous in their C-termini; however, the human orthologue contains a unique N-terminus (33).

The PKA RII binding region of AKAP220 was narrowed to the common C-terminal region of the protein (33, 68). In vitro assays using a recombinant rat AKAP220 fragment containing amino acid residues 761 through 1129 confirmed that it bound to RII$\alpha$ by overlay (68). Sequence comparison to AKAP150 and Ht31 narrowed the amphipathic helix to residues 905 through 918. In vivo evidence for an AKAP220-RII interaction was determined by copurification of PKA catalytic activity with AKAP220 immunoprecipitates from rat testis lysate. Its specificity was confirmed upon inhibition in the presence of PKI (68). Furthermore, indirect immunofluorescence of AKAP220 and RII confirmed distinct yet overlapping patterns in the TM4 rat testis cell line (68). In contrast, human AKAP220 coimmunoprecipitated both type I and type II PKA regulatory subunits, which demonstrates the potential to tether both types of PKA within the cell (33).

The targeting domain of AKAP220 is not completely clear, but in rat it potentially consists of a peroxisomal targeting motif (68). The final three amino acid residues of rat AKAP220 are cysteine, arginine, and leucine (CRL), which satisfies the criteria for a C-terminal peroxisomal targeting signal (69–71). Indirect immunofluorescence of AKAP220 and a peroxisomal marker in the TM3 and TM4 rat testis cell lines showed similar punctate staining patterns (68). These data suggest that AKAP220 may associate with a portion of peroxisomes.

Alternatively, human AKAP220 protein does not contain the C-terminal CRL motif (33). Indirect immunofluorescence localized human AKAP220 to the cytoplasm of premeiotic spermatocytes and colocalized AKAP220, RII$\alpha$, and RI$\alpha$ to the midpiece/centrosome region of postmeiotic germ cells and mature sperm. These data demonstrate variable AKAP220 localization depending upon developmental stage. This perhaps indicates the requirement of an additional targeting protein whose expression is developmentally regulated, as similarly proposed for mAKAP targeting (37). Furthermore, AKAP220 could not be isolated by detergent extraction of sperm tails, which suggests a possible association with cytoskeletal structures (33). This is in contrast to another midpiece-associated dual function AKAP, S-AKAP84/D-AKAP1, that was soluble. Consequently, human AKAP220 subcellular localization may depend upon cell type and developmental stage.

The discrepancy between rat and human subcellular targeting domains is somewhat surprising considering that other AKAP orthologues retain similar targeting domains. Since the CRL motif of rat AKAP220 was not tested rigorously to determine whether it was indeed the subcellular targeting domain, it is possible that it may not be important for AKAP220 function in vivo. Thus, another as yet unidentified targeting sequence common to both rat and human AKAP220 may exist. A more plausible speculation is that alternative splicing occurs with the AKAP220 gene. This idea is supported by the detection of three AKAP220 mRNA transcripts. Consequently, different targeting domains could be present in the various splice forms, and localization may depend upon the spatial and temporal expression of each form.

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## AKAP220 Signaling Complexes

Evidence that AKAP220 is a platform for a multivalent signaling complex was found when it was recognized that the human protein contains an R/KVXF (single letter amino acid code) motif. This loosely defined motif is a consensus site for association with PP1 (72, 73). Based on this evidence a variety of human AKAP220 fragments were used in a PP1 overlay assay to test for binding. A fragment encompassing residues 385 through 530, including the R/KVXF motif, bound to the phosphatase (41). Surface plasmon resonance spectroscopy revealed that recombinant PP1c$\alpha$ bound to this fragment with nanomolar affinity, while the analogous rat fragment bound with 20-fold lower affinity (41). Further in vitro mapping studies yielded additional binding determinants in the C-terminus between residues 1711 and 1901 of human AKAP220 (74). In vivo interaction was confirmed when precipitation of PP1$\alpha$ from rat brain extract by microcystin-sepharose affinity resulted in coprecipitation of AKAP220. In addition, confocal analysis of AKAP220, PP1$\gamma$, and RII in rat hippocampal neurons demonstrated overlapping signals in the cell body (41). Thus, a variety of in vitro and in vivo experiments support the notion that AKAP220 can simultaneously associate with PP1 and RII.

Experiments testing functional activity showed that PP1$\beta$ immunoprecipitates from rat testis were enriched in specific PKA activity (41). Conversely, AKAP220 is a competitive inhibitor of PP1 activity as shown by in vitro phosphatase assays (74) (Figure 3). Residues 1711 through 1901, which also contain PP1 binding determinants, were required to maintain PP1 in an inactive state (74). The ability of AKAP220 to inhibit PP1 was further enhanced by anchoring of the PKA RII subunit. Furthermore, RII still enhanced PP1 inhibition when tested with an AKAP220-RII binding mutant, which suggests that RII directly binds to PP1 (74). These experiments imply that the molecular mechanism of phosphatase inhibition by AKAP220 and RII involves separate binding sites on the phosphatase. Thus, AKAP220 and RII appear to work in an additive manner to inhibit PP1 (74). Other anchoring proteins such as gravin and AKAP79 also bind to and inhibit their anchored enzymes (42, 75, 76). Thus, there is a precedent that AKAPs not only can provide substrate specificity by subcellular localization, but also can render an additional level of regulation by directly affecting the activity of an anchored enzyme.

## AKAP 350/450/CG-NAP/YOTIAO

Coordination of multivalent signaling complexes has been described for a family of alternatively spliced AKAPs encoded by a single gene of 50 exons on 7q21 (77, 78). These splice forms include at least two nearly identical forms of AKAP350 (77, 79), AKAP450 (78), CG-NAP (80), and yotiao (81). AKAP350 was first identified by RII overlay from purified centrosomes isolated from the human lymphoblastic cell line KE37 (79). It was independently identified as AKAP450 by RII overlay screening of a Jurkat T lymphocyte expression library (78) and as CG-NAP (Centrosome and Golgi localized PKN-associated protein) in a yeast two-hybrid

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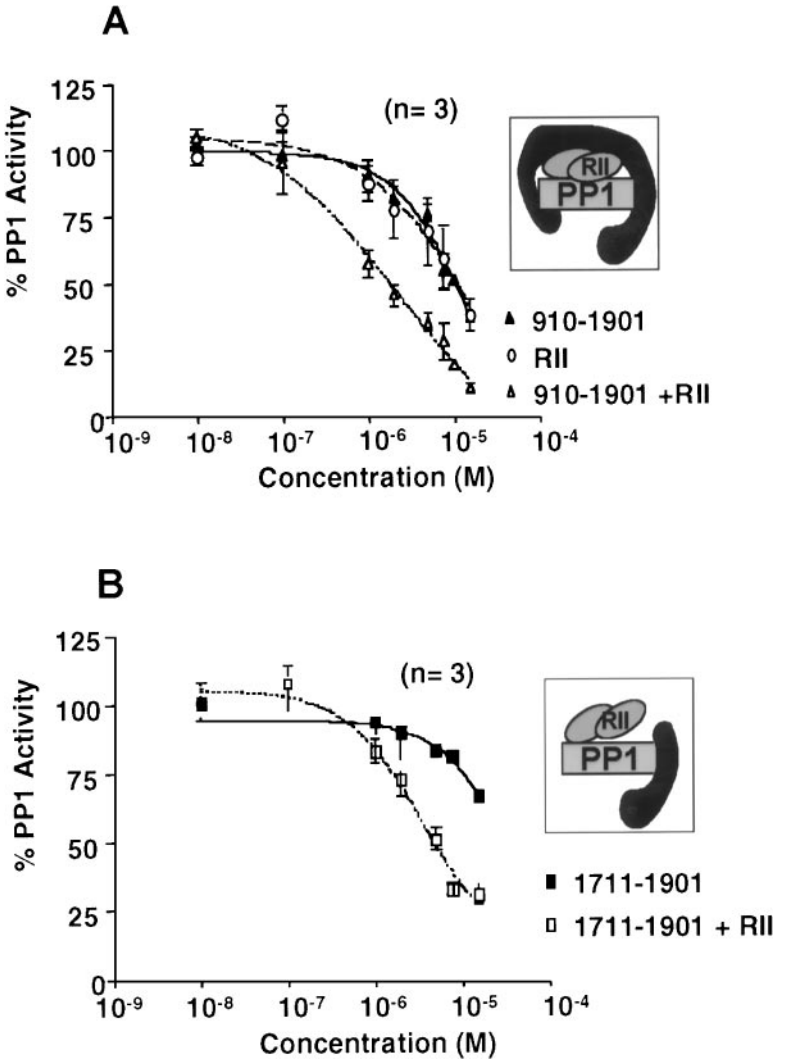


**Figure 3** RII binding enhances PP1 inhibition by AKAP220. The inhibitory properties of selected AKAP220 fragments toward PP1$\alpha$ were measured in the presence of RII. (*A*) Dose-response curves for AKAP220(910-1901) fragment (closed triangles), RII (open circles), and AKAP220(910-1901) fragment plus RII (open triangles) are presented. Inset demonstrates the potential role of AKAP220 to coordinate RII-anchoring in a manner that optimizes inhibitory contact with the phosphatase. (*B*) Dose-response curves for the AKAP220(1711-1901) fragment in the presence (open squares) and absence of RII (closed squares). Inset depicts how RII and the AKAP220 fragment act synergistically to inhibit the phosphatase. Reprinted with permission of the *Journal of Biological Chemistry* (74).

assay for PKN-interacting proteins (80). For simplicity, AKAP350/450/CG-NAP is referred to as AKAP350 throughout this review and yotiao is discussed separately.

## AKAP 350/450/CG-NAP Coordinated Signaling Complexes

Northern analysis of AKAP350 demonstrated a wide tissue distribution with 9.5-kb and 11-kb messages predominant in kidney and skeletal muscle, a 9.5-kb message in liver, and an 11-kb message in heart and brain (77). The AKAP350 protein product is predicted to be a coiled-coil protein with the unique property of containing two putative RII binding regions at residues 1438–1455 and 2540–2558 (80). Though insoluble, AKAP350 could be extracted in part with the RII subunit of PKA (79), and it colocalized with RII in vivo when analyzed by indirect immunofluorescence (80). Furthermore, two AKAP350 deletion mutants separately encompassing each RII binding site were able to interact with RII in cells under overexpression conditions (80). This potentially confers to AKAP350 the ability to coordinate two PKA holoenzymes in one signaling complex.

Though the functional roles of PKA anchoring by AKAP350 are not certain, its subcellular localization may provide a clue. Localization studies showed that the C-terminal 266 amino acid residues fused to GFP were sufficient to localize GFP to the centrosome (82). Other studies confirmed these findings using indirect immunofluorescence and extended them by observing that AKAP350 centrosomal localization is constant during the entire cell cycle (77, 80). Purely based on this data, functional events mediated by AKAP350 may include some role in microtubule nucleation or in cell cycle progression.

These data also indicate that the targeting domain of AKAP350 resides in its C-terminus. Database search revealed that this C-terminal region contains at least 38% homology to the C-terminus of pericentrin (77, 78, 80, 82), another centrosomal AKAP (83). This common domain was dubbed the PACT domain (pericentrin-AKAP450 centrosomal targeting domain), and its overexpression was able to displace pericentrin from the centrosome (82). This suggests that AKAP350 and pericentrin may compete for the same binding site within the centrosomal region. Thus, the levels of AKAP protein expressed may dictate which signaling complex is located at this particular site. Alternatively, it may be that AKAP350 and pericentrin are present at the same site in cells to perform a coordinated function.

Finally, two studies also observed AKAP350 at the Golgi in interphase cells (78, 80), whereas another saw additional discrete foci in the cleavage furrow of dividing HCT116 cells during anaphase and telophase (77). This suggests that AKAP350 may be required for events such as intracellular membrane trafficking and formation of the contractile ring for cytokinesis. However, since colocalization of RII with AKAP350 was not observed in the cleavage furrow, this particular function appears independent of PKA activity (77).

In addition to PKA, AKAP350 can interact with multiple signaling enzymes such as PKN, PP1, and PP2A (80). PKN is a serine/threonine kinase that associates

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with and phosphorylates intermediate filament proteins in vitro (84, 85). Thus, coordination of PKN by AKAP350 signaling complexes could be important for cytoskeletal reorganization events. Activation of PKN is achieved by the small GTPase Rho (86–88), by unsaturated fatty acids (89, 90), or by truncation of its N-terminal regulatory region (89–91). Interestingly, PKN binds to AKAP350 through its N-terminal domain (80). The fact that PKN is activated upon truncation of its N-terminus may imply that AKAP350 can influence the catalytic state of PKN. Finally, the C-terminus of AKAP350 was shown to interact with the PP2A regulatory B subunit PR130, while a conventional R/KVXF motif at residues 1053 through 1056 provides the PP1-interacting determinant (80).

AKAP350 also associates with immature hypophosphorylated PKC$\varepsilon$ at the golgi/centrosome area (92). PKC requires three phosphorylation events in order to respond to second messenger signals and become active (93–96). One site is in the activation loop, and two sites are C-terminal to the kinase domain (97, 98). The activation loop of PKC, as well as that of PKB, is phosphorylated by the constitutively active phosphoinositide-dependent protein kinase 1 (PDK1) (99–103). In the case of PKB, its activation loop phosphorylation site is not available for phosphorylation by PDK1 until binding of phosphatidylinositol-(3,4,5)-triphosphate to its pleckstrin homology domain. This interaction causes PKB translocation to the plasma membrane and induces a conformational change that exposes the phosphorylation site (104). One study suggested that PKC$\varepsilon$ activation may occur in an analogous manner (92). Thus, AKAP350 may act as a scaffold to direct the phosphorylation events required for PKC$\varepsilon$ maturation. Alternatively, AKAP350 could simply protect the unstable premature protein from degradation (92). Thus, it is clear that AKAP350 provides a point of integration for several distinct signaling pathways to mediate a variety of physiological functions.

## Yotiao and NMDA Receptor Function

Yotiao is the shortest splice variant of the AKAP350 locus. It was first identified in a yeast two-hybrid screen for NR1 interacting proteins using the C-terminal 105 amino acid residues of NR1A, a subunit of the NMDA receptor (81). NMDA receptors are a subclass of glutamate receptors found in the postsynaptic density (reviewed in 105) that participate in synaptic plasticity (106, 107), neural development, and excitotoxicity (108, 109). The receptors are heteromultimers composed of an NR1 subunit and a variety of NR2 family members (110–114). Yotiao specifically interacts with the splice form of NR1 that contains the C1 exon (40, 81).

Northern analysis detected yotiao mRNA transcript in a variety of tissues including heart, brain, placenta, skeletal muscle, and pancreas (81). However, the probes used to detect the transcript included sequences common to the other AKAP350 splice variants. Thus, the tissue distribution observed most likely is not unique to yotiao but reflects the entire family. Yotiao protein was predominantly associated with the membrane fraction generated from rat brain, which is consistent with an interaction between yotiao and NR1 at synaptic sites (81). Immunohistochemical

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experiments in rat brain demonstrated colocalization of yotiao and NR1; however, the immunostaining patterns were not completely overlapping (81). This suggests that yotiao and NR1 are not exclusive partners, consistent with the fact that not all NR1 subunits of NMDA receptors contain the C1 exon cassette required for interaction.

Yotiao's property as an AKAP was later identified when it was cloned from a pituitary cDNA expression library in a screen for RII interacting proteins (40) and from a rat thyroid cDNA library using mouse RII$\alpha$ as a bait in a yeast two-hybrid screen (115). Yotiao immunoprecipitates from rat brain extract contained RII subunit and exhibited specific PKA catalytic subunit activity (40). Mapping studies determined that residues 1440 through 1457 of yotiao composed the RII binding site.

Activation of PKA enhances current through NMDA receptors (116, 117). The functional consequence of yotiao-anchored PKA activity on NMDA function was determined by whole cell current recordings of transfected cells (40). When cells expressing C1 exon containing NR1A and yotiao were subjected to a cell permeant analog of cAMP, current recordings were enhanced over controls in which yotiao was absent. Furthermore, the presence of Ht31 peptide or the PKA inhibitor PKI abolished the augmentation, which demonstrated the requirement of a yotiao-anchored pool of PKA for efficient generation of current through NMDA receptors (40).

PP1 activity is also an important regulator of NMDA receptors (118–120). Immunoprecipitation experiments demonstrated copurification of yotiao and phosphatase PP1 from rat brain extracts (40). Though an R/KVXF PP1 targeting motif does exist in yotiao, it is not essential for its interaction. Instead, in vitro experiments demonstrated that residues 1171 through 1229 of yotiao were involved in PP1 binding (40). Phosphatase assays demonstrated that yotiao did not inhibit PP1 activity toward NR1A substrate (40). Thus unlike AKAP220, which maintains anchored PP1 in an inactive state (74), yotiao may target active PP1 to the NMDA receptor.

Functional studies support the idea that yotiao-anchored PP1 activity regulates the NMDA receptor. Whole cell recordings made from cells transfected with NR1A and yotiao were tested in the presence of the PP1 inhibitor Gm peptide (40). A greater time-dependent increase in NMDA receptor currents was observed in these cells as compared to those not expressing yotiao. This demonstrates the impact of a yotiao-anchored pool of PP1.

A molecular model for NMDA receptor regulation by yotiao anchored PKA and PP1 was proposed by Westphal et al. (40) (Figure 4). Under resting conditions, active PP1 may favor dephosphorylated channels. Upon cAMP stimulation, PKA is activated to phosphorylate nearby NMDA receptors, overcoming basal levels of PP1 activity. This enhances the current through the NMDA channels. PP1 dephosphorylation of the receptors resets the channels into a deactivated state. Thus, coordination of PP1 and PKA by yotiao mediates the localization of the opposing functions required to modulate NMDA receptor function (40, 81).

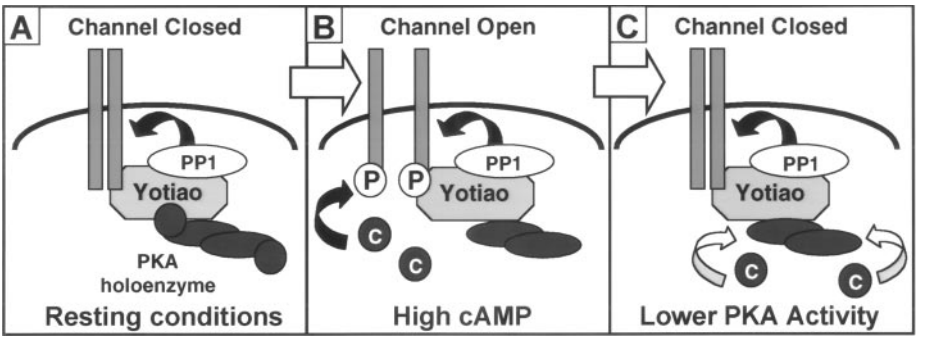


**Figure 4** A molecular model for NMDA receptor regulation by yotiao-anchored PKA and PP1. (*A*) Under resting conditions, active PP1 may favor dephosphorylated channels. (*B*) Upon cAMP stimulation, PKA catalytic subunit is released and phosphorylates NMDA receptors, overcoming PP1 basal activity. This allows the channels to open and enhances the current through NMDA channels. (*C*) Subsidence of cAMP pools allows reformation of PKA holoenzyme, and PP1 dephosphorylation of NMDA receptors resets the channels into a closed, deactivated state.

## CONCLUSION AND PERSPECTIVES

The many ways in which AKAPs create intracellular signaling specificity are highly sophisticated. Compartmentalization of PKA by AKAPs provides an important molecular mechanism to ensure specific activation and appropriate substrate selection. Coordination of several signaling enzymes within one macromolecular complex allows AKAPs to integrate several different transduction pathways. However, while we have made great strides to dissect the molecular composition of these signal transduction units, we are still on the cusp of understanding the biological functions they mediate. Thus, the next challenge is to investigate the precise functional roles of AKAPs in physiological processes.

In the case of mAKAP, future direction could include functional studies into the influence of RyR2 function by phosphodiesterases. For example, could local PDE inhibition or disruption of an SR-associated pool of PDE be a reason for PKA hyperactivity in failing heart tissue? Or more specifically, is there an mAKAP-anchored PDE activity associated with the ryanodine receptor? Answering these questions could aid in the understanding of molecular aberrations contributing to heart failure. Furthermore, the role for mAKAP signaling complexes may not be limited to the heart. In the EST database there are at least 12 mouse and 27 human ESTs from a variety of sources including brain, retina, testis, and neuronal tissue. Thus, the full extent of mAKAP's role in mediating the localization of signaling complexes may not be fully appreciated until the generation of a genetic model.

There are also many issues concerning AKAP220 and AKAP350 anchoring that should be addressed. For AKAP220, it would be interesting to determine if

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alternative splicing accounts for the divergence between rat and human sequences. This could be coupled with additional mapping studies to conclusively determine the targeting domain directing AKAP220 subcellular localization. Additionally, studies are warranted that would explore the molecular mechanisms of AKAP220- and AKAP350-anchored signaling complexes and their functional consequences in a physiological context. Future experiments could include proteomics approaches to identify substrates acted upon by the anchored enzyme components. Genetic models would also be useful in understanding the importance of AKAP220 and AKAP350 function in vivo. Finally, given the complexity of AKAP350 due to its many splice variants, it would be interesting to determine the contribution of each one to a biological process. For example, like yotiao, AKAP350 contains the interaction domains for PP1 and NR1A (40). Does AKAP350 play a role in NMDA receptor function, or does its tissue expression and cellular localization preclude it from ever interacting with these channels? Do AKAP350 and pericentrin segregate pathways within the same centrosomal compartment or are their actions coordinated to perform one function? These and many other questions still remain to be answered.

We anticipate that a multitude of new reagents will be used to address these issues. By far, the Ht31 peptide has been the most useful tool in understanding the importance of compartmentalized pools of PKA. However, since Ht31 globally disrupts the PKA/AKAP interaction, it will be advantageous to develop reagents that specifically delocalize a particular AKAP. These could be used in functional studies aimed at dissecting the importance of distinct AKAP-anchored pools of signaling enzymes. Finally, a variety of genetic approaches to generate relevant biological models will contribute significantly to the understanding of how AKAPs mediate the execution of many physiological processes. Thus, this new era of reagents undoubtedly will be instrumental in defining the functional roles of AKAPs in vivo.

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