AKAP79 INTERACTS with MULTIPLE ADENYL CYCLASE (AC) ISOFORMS and SCAFFOLDS AC 5 and 6 to AMPA RECEPTORS

Riad Efendiev1, Bret K. Samelson2, Bao T. Nguyen1, Prasad V. Phatarpek1, Faiza Baameur1, John D. Scott2, and Carmen W. Dessauer1*

From the 1Department of Integrative Biology and Pharmacology, University of Texas Health Science Center at Houston, 6431 Fannin St, Houston, Texas 77030 and the 2Howard Hughes Medical Institute, Department of Pharmacology, Box 357750 University of Washington, School of Medicine, Seattle, Washington 98195

Running title: AKAP79 Binds and Regulates Multiple AC Isoforms

*Address correspondence to: Carmen W. Dessauer, Department of Integrative Biology and Pharmacology, University of Texas Health Science Center at Houston, 6431 Fannin St, Houston, Texas 77030; Tel: (713) 500-6308; Fax: (713) 500-7444; E-mail: Carmen.W.Dessauer@uth.tmc.edu

Spatiotemporal specificity of cAMP action is best explained by targeting protein kinase A (PKA) to its substrates by A-Kinase Anchoring Proteins (AKAPs). At synapses in the brain AKAP79/150 incorporates PKA and other regulatory enzymes into signal transduction networks that include β-adrenergic receptors, α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) and N-methyl-D-aspartic acid (NMDA) receptors. We previously showed that AKAP79/150 clusters PKA with type 5 adenylyl cyclase (AC5) to assemble a negative feedback loop in which the anchored kinase phosphorylates AC5 to dynamically suppress cAMP synthesis. We now show that AKAP79 can associate with multiple AC isoforms. The N-terminal regions of AC 5, 6, and 9 mediate this protein-protein interaction. Mapping studies located a reciprocal binding surface between residues 77-108 of AKAP79. Intensity- and lifetime-based Fluorescence Resonance Energy Transfer (FRET) demonstrated that deletion of AKAP7977-108 region abolished AC5/AKAP79 interaction in living cells. Addition of the AKAP7977-153 polypeptide fragment uncouples AC5/6 interactions with the anchoring protein and prevents PKA-mediated inhibition of AC activity in membranes. Use of the AKAP7977-153 polypeptide fragment in brain extracts from wild type and AKAP150-/- mice reveals that loss of the anchoring protein results in decreased AMPA receptor-associated AC activity. Thus we propose that AKAP79/150 mediates protein-protein interactions that place AC5 in proximity to synaptic AMPA receptors.

Localized activation of PKA triggers a plethora of intracellular signaling processes (1). Precise control of these phosphorylation events is often achieved by restricted activation of PKA in discrete microenvironments. AKAPs participate in this process by tethering the kinase close to preferred substrates. AKAPs now represent a family of 43 diverse but functionally related proteins that bind the regulatory subunit dimer of the PKA holoenzyme (2).

AKAPs have been identified in a range of species, tissues and cellular compartments. The AKAP79/150 group of anchoring proteins is perhaps the best understood member of this class of signal organizing proteins. AKAP79/150 consists of three orthologs: bovine AKAP75, human AKAP79 and murine AKAP150. Although originally identified in the postsynaptic densities of neurons, this group of anchoring proteins is also expressed in a variety of other tissues. In addition to binding PKA, AKAP79 has the ability to bind protein phosphatase 2B (3) and protein kinase C (PKC) (4,5). By organizing these signal transduction and signal termination enzymes in the same location, AKAP79 provides a platform to facilitate the bi-directional control of cAMP and calcium mediated signaling events.

Although anchoring of PKA with its substrates provides an efficient mechanism for the spatial regulation necessary for selectivity of cAMP signaling, it was not clear how local pools of cAMP are managed. We have shown that AC isoforms can specifically interact with three different AKAP complexes, AKAP79, Yotiao, and mAKAP, to regulate events downstream of cAMP production (6-8). We have also demonstrated that anchoring of AC5 to an AKAP79/150 complex...
provides negative feedback on AC5 via PKA phosphorylation of AC5 within the complex (6).

Although characterization of the AKAP79-AC5 interaction has shed some light on the advantages gained by localizing different components of cAMP signaling pathways, several key issues remain unresolved. First of all, do other AC isoforms interact with AKAP79 or other anchoring proteins? Secondly, are AC isoforms recruited into larger signaling networks via their protein-protein interactions with AKAP79? AKAP79/150 has been shown to form a multi-protein signaling complex with AMPA and NMDA receptors (9-11), adhesion molecules (12) and cytoskeleton proteins (13), all of which play important roles in synaptic function. Is AC an integral part of this complex, and if so, which AC isoforms dominate?

In this report we demonstrate that AKAP79 interacts with a distinct subset of AC isoforms. The interaction of AKAP79 with AC5 is direct, involving the N-terminus of AC5 and residues 77-108 of AKAP79. Finally, disruption of the AKAP79 and AC5 complex results in a significant decrease in AMPA receptor type 1 subunit (GluR1)-associated brain AC activity.

**EXPERIMENTAL PROCEDURES**

**Plasmids—**Plasmids AKAP79-GFP-pcDNA3, AKAP79-GFP-pcDNA3-1-ΔB-153 and purified recombinant H6-S-tag-AKAP79 polypeptide fragments (77-153) and (109-290) were previously described (14). Na⁺,K⁺-ATPase (NKA) α1-YFP plasmid and protein characterization was previously described in (15). CFP-tagged AKAP79 was created by subcloning of enhanced cyan fluorescence protein (CFP) into pEGFPN1-AKAP79 vector using the restriction enzymes BamHI and NotI. AKAP79-Myc-pScript polypeptide fragments (1-153, 77-153 and 154-427) were constructed by PCR. AC5-YFP-pcDNA3 was constructed in a two step procedure by first, generating a unique KpnI restriction site by PCR immediately prior to the stop codon of human AC5 in hAC5-pcDNA3 and second, by subcloning in the yellow fluorescence protein (YFP) using the restriction enzymes KpnI and BamHI. Sequences of all modified fragments were confirmed by restriction digests and DNA sequencing.

**Antibodies—**Antibodies used were mouse α-Myc (Santa Cruz), mouse α-GST (Invitrogen), mouse α-AKAP79 (Transduction Lab), mouse α-FLAG M2-Agarose (Sigma), mouse α-S-tag (Novagen), rabbit α-GluR1 (Millipore) and rabbit α-AKAP150 (VO88).

**Transfections—**Human embryonic kidney (HEK293) cells were seeded at 5 x 10⁶ cells per 10 cm culture dish and grown for 1 day in DMEM with 10% FBS prior to transfection with Lipofectamine 2000 (Invitrogen) or jetPEI (Polyplus). Plasmid DNA (10 µg total) used to transfect cells in 10 cm dishes included: AKAP79 (5 µg), AC3 (3 µg), AC5 (2.5 µg), all other ACs (5 µg), and pcDNA3. Cells were used 44-50 h after transfection.

**Protein Purification and Glutathione S-Transferase (GST) pull-down assay—**Gαs-H6 was expressed in *E. coli* and activated with [³⁵S]GTPγS (16). GST-tagged proteins were purified on glutathione resin (Amersham) in buffers lacking detergents. GST pull-down assays were as previously described (17), utilizing lysates from transfected HEK293 cells (500 µg) or the indicated purified polypeptides (1 µM).

**Immunoprecipitation of Adenylyl Cyclase Activity—**Immunoprecipitations using anti-Flag agarose followed by measurement of associated AC activity (IP-AC assays) were performed as described (6). Preparation of brain tissue extracts and subsequent IP-AC assays were performed essentially as described (8). Competing polypeptides were added prior to homogenization. AKAP150 (2 µg) and GluR1 (0.4 µg) antibodies were utilized followed by pull-down of complexes with protein A sepharose (30 µl of 50% slurry) and measurement of AC activity. All measurements were performed in duplicate and were normalized to IgG samples with control polypeptide.

**Membrane Preparation and Adenylyl Cyclase Activity Measurements—**Membranes from transfected HEK293 cells were prepared as described (8). Membranes (30 µg per reaction) were then immediately assayed for adenylyl cyclase activity upon stimulation with the indicated reagents (18).

**FRET Experiments—**HEK293 cells were plated on cover slips in six-well dishes at 10% confluence. The next day, cells were transfected with 1.8 µg of total DNA, which included 0.4 µg of wild type or mutant AC5-YFP, 1 µg of
AKAP79-CFP and 0.4 µg pcDNA3. Before imaging, media was replaced with Tyrode's buffer (10 mM HEPES, pH 7.4, 145 mM NaCl, 4 mM KCl, 1 mM CaCl2, 1 mM MgCl2, and 10 mM glucose). Fluorescence images were acquired after 48 h of transfection using a microscope (TE 2000; Nikon, Tokyo, Japan) with a DG4 xenon light source and two CoolSNAP cameras (Roper Scientific, Trenton, NJ). For FRET determinations, a z-stack of at least 7 xy-planes of three images were acquired sequentially (exposure time, 200 ms) using the following filter sets: donor (CFP; excitation, 436/20 nm; emission, 465/30 nm), FRET (CFP/YFP; excitation, 436/20 nm; emission, 535/30 nm) and acceptor (YFP; excitation, 500/20 nm; emission, 535/30 nm). Deconvolution of z-stacks was performed using Slidebook software. Corrected, sensitized FRET (FRETc) was calculated using the equation FRETc = IFRET - (a × ICFP) - (b × IYFP), where IFRET, ICFP, and IYFP correspond to background-subtracted images of cells expressing both CFP and YFP acquired through the FRET, CFP, and YFP channels, respectively. The values a and b are the bleed-through values of CFP and YFP in FRET channel, respectively. Calibrations of bleed-through were performed in cells expressing either CFP- or YFP-tagged proteins and were calculated as 0.54 (a) and 0.04 (b) for CFP and YFP, respectively. In cells expressing both CFP and YFP-tagged proteins, FRET values were calculated according to the methods: FRETc/ICFP (19), FRETc = FRETc/(ICFP × IYFP) (20) and N FRET = FRETc/SQRT(ICFP × IYFP) (21). Pseudocolor FRETc/ICFP images were obtained using the Slidebook software and are displayed with deep blue indicating low values and bright red indicating high values of FRET. For fluorescence images, figures show representative images from 16 to 22 different cells from four different experiments.

**RESULTS**

AC 2, 3, 5, 6, 8 and 9 associate with AKAP79—Various AC isoforms were screened for association with AKAP79 in an over-expression system. HEK293 cells were transiently transfected with vector, Flag-tagged AKAP79, and/or AC 1-9. AKAP79 was immunoprecipitated from cell lysates using anti-Flag agarose. AC activity was measured in each immune complex (referred to as an IP-AC assay) for detection of direct or indirect binding of AC. For all isoforms except AC9, the immunoprecipitates were stimulated with activated Gαs and forskolin (Fig. 1A). Since AC9 is not sensitive to forskolin, a higher concentration of Gαs was used in these samples (Fig. 1B). AC activity in the cell lysates confirmed that each AC isoform was expressed over background AC activity (Sup. Fig. 1). As shown in figures 1A and B, the AC 2, 3, 5, 6 and 9 isoforms associate with AKAP79. AC8 activity was consistently higher in immunoprecipitates of AC8 + AKAP79 versus AC8 alone when assayed with Gαs and forskolin.
although the change was not statistically significant. Stimulation with Ca\(^{2+}\)/calmodulin (CaM), a potent activator of AC8 resulted in a significant association of AC8 activity with AKAP79 (Fig. 1C).

**AKAP79 inhibits the activity of AC 2, 5 and 6 in isolated membranes**—To determine the effect of AKAP79 on AC activity, HEK293 cells were transfected with pcDNA3 vector and AC 2, 3, 5, 6 or 9 ± AKAP79. Membranes were isolated to measure AC activity. AKAP79 had no effect on the basal activity of any isoform (data not shown). Membranes containing AC 2, 5 and 6 were inhibited in the presence of AKAP79 when stimulated with activated G\(\alpha_s\) (Fig. 2A). Membranes containing AC9 were not inhibited in the presence of AKAP79 when stimulated with a higher concentration of G\(\alpha_s\) (Fig. 2B). AC activity was also inhibited in membranes when stimulated by G\(\alpha_s\) + G\(\beta\gamma\) (AC2, 46% and AC5, 73% inhibition), forskolin alone (AC5, 38% inhibition), or G\(\alpha_s\) + forskolin (AC2, 39%, AC5, 42% and AC6, 28% inhibition) (data not shown). Under all stimulation conditions tested AC3 activity was unaffected by AKAP79 expression.

**N-termini of AC 5, 6, and 9 mediate interactions with AKAP79**—We have previously shown that the N-terminus of AC2 bound the AKAP Yotiao (8). We therefore screened GST-tagged AC N-termini (AC-NT) from all AKAP79-inhibited AC isoforms for interactions with this anchoring protein by GST pull-down assay. AC 5, 6 and 9 NT, but not AC 2 or 3 NT, bound AKAP79 (Fig. 3A).

GST pull-down assays were used to map the AC5 binding site on AKAP79. Cell lysates from HEK293 cells expressing various Myc-tagged truncations of AKAP79 or purified truncations of AKAP79 were incubated with GST-AC5-NT (Fig. 3B). The first 153 residues of AKAP79 and a region that encompasses residues 77-153, bind GST-AC5-NT but not GST alone (Fig. 3C). The AKAP79\(^{77-153}\) polypeptide fragment comprises the 2\(^{nd}\) (B) and 3\(^{rd}\) (C) polybasic regions of AKAP79, however neither region alone is sufficient to tether the protein to the plasma membrane (3). To more definitively map the binding site for AC5, we incubated E. coli purified polypeptide fragments encompassing residues 77-153 and 109-290 of the anchoring protein with a GST-tagged N-terminal region of AC5. AKAP79\(^{77-153}\), but not AKAP79\(^{109-290}\) directly binds AC5-NT (Fig. 3D). However, binding is greatly reduced when experiments were repeated with a truncated form of AC5 that lacked the first 60 aa (GST-AC5\(\Delta\)60-NT). Based on the mapping depicted in figure 3B, we conclude that the first 60 aa of AC5 bind to the second polybasic region of AKAP79.

**AKAP79\(^{77-153}\) is a selective disruptor of AC-AKAP79 interactions**—To determine if AKAP79\(^{77-153}\) can be used as a selective disruptor of AKAP79-AC binding, we performed IP-AC assays in the presence of AKAP79\(^{77-153}\) or a control polypeptide AKAP79\(^{109-290}\). Addition of AKAP79\(^{77-153}\) disrupted association of AC 5 and 6 with AKAP79, but not that of AC 2 or 9 (Fig. 4A). This result suggests that that AKAP79\(^{77-153}\) selectively disrupts AC5/6 binding to AKAP79 and infers that the AC 2 and 9 isoforms interact with different regions of the anchoring protein.

In order to focus our study, the remainder of the experiments concentrates on the AC5-AKAP79/150 interaction. We have previously shown that anchored PKA phosphorylated AC5/6 to suppress cAMP synthesis (6). In agreement with this finding we were able to demonstrate that AC5 activity is inhibited upon co-expression with AKAP79 when stimulated with G\(\alpha_s\) (Fig. 2A and Fig. 4B). To determine if inhibition of AC5 is reversible, we utilized the AKAP79\(^{77-153}\) polypeptide to disrupt AC5-AKAP79 protein-protein interactions. Membranes prepared from HEK293 cells expressing AC5 +/- AKAP79 were assayed for G\(\alpha_s\)/forskolin-stimulated AC activity in the presence of purified AC-AKAP disruptor fragment (Fig. 4B and C). AKAP79\(^{77-153}\) reversed AKAP79-mediated inhibition of AC5 (IC\(_{50}\) = 1.0 µM). Control experiments confirmed that the AC-AKAP disruptor had no effect on AC5 in the absence of AKAP79.

**FRET between AC5 and AKAP79**—To explore the dynamics of this protein-protein interaction inside cells, we generated fluorescence tagged versions of each protein. Fluorescent probes containing YFP at the C terminus of AC5 (AC5-YFP), and CFP at the C terminus of AKAP79 (AKAP79-CFP) were produced. In addition we constructed a mutant form of AKAP79 lacking aa 77-108 fused to CFP (AKAP79AB-CFP). Initial experiments confirmed that fusion of
the fluorescent tags did not alter the AC5 activity with or without AKAP79 (Fig 5A). Activity measurements confirmed that expression of AC5-YFP stimulated cAMP synthesis and co-expression with AKAP79-CFP attenuated this response (Fig 5A). In addition, AC5-YFP was localized to the plasma membrane when expressed in HEK293 cells at low levels (Fig. 5B). Higher levels of expression generally produced a particulate cytoplasmic fluorescence that was excluded from the nucleus. Therefore, we have titrated AC expression to levels that produce a largely plasma membrane expression pattern for all fluorescence and activity assays. Previous reports have suggested that AC5 may be present on the nuclear envelope in cardiac myocytes (24). However, we detected no such localization upon expression in HEK293 cells. To analyze AKAP79 interactions with AC5 in living cells, we used an intensity-based FRET approach employing AC5-YFP and AKAP79-CFP. There is a strong inverse distance relationship between FRET and chromophore separation, such that FRET between the donor CFP and acceptor YFP molecules only occurs if the two proteins are in proximity (<10 nm). Numerous mathematical methods are used to quantify FRET. We compared the three most commonly used methods FRET^ICFP (19), FRET^N (20) and N^FRET (21). The PKA RII subunit tagged with YFP at C-terminus was used as an established FRET partner for AKAP79-CFP, while AKAP79 lacking a PKA-binding site (AKAP79ΔPKA-CFP) was used as a negative control (25) (Fig. 5C). A second negative control for non-specific membrane protein interactions was the transmembrane protein NKAα1-YFP. Any FRET signal between AKAP79-CFP and NKAα1-YFP or PKA-RII-YFP and AKAP79ΔPKA-CFP set the baseline for non-specific membrane interactions. All three computational methods show significant FRET at the plasma membrane for AC5-YFP and AKAP79-CFP relative to all negative controls and that is lost upon deletion of the second polybasic region of AKAP79 (AKAP79ΔB-CFP) (Fig. 5 B and C and Sup. Table 1).

FLIM-FRET between AC5 and AKAP79–To complement the intensity-based FRET analysis, we performed FLIM-FRET of HEK293 cells expressing AC5-YFP and AKAP79-CFP. When two chromophores undergo energy transfer, the lifetime of the donor chromophore (CFP) decreases in the presence of an acceptor (YFP) and provides a more quantitative measurement of FRET efficiency (F_{eff}) (22). We observed a significant decrease in CFP lifetime in cells expressing both AC5-YFP and AKAP79-CFP, as compared to those expressing AKAP79-CFP alone (2.26±0.01 vs. 2.78±0.01 ns; F_{eff} = 19.2±1.1%, mean ± S.E.M.) (Fig. 6A). No decrease in CFP lifetime was observed in cells expressing AC5-YFP and AKAP79ΔB-CFP compared with AKAP79ΔB-CFP alone (2.81±0.01 vs. 2.77±0.01) (Fig. 6B). Control experiments monitored FLIM-FRET of AKAP79-CFP with its well-characterized binding partner the RII subunit of PKA fused to YFP (F_{eff} = 23.8±1.3%). A negative control for this study was AKAP79ΔPKA-CFP, a form of the anchoring protein that lacks the PKA anchoring domain (Fig. 6A and Sup. Fig. 2). Taken together the data presented in figure 6 provides compelling evidence that AKAP79 functions to dynamically interact with AC5 in living cells.

AMPA receptor associated AC activity is mediated in part by AKAP150 and AC5/6 interactions–Finally, we investigated the physiological relevance of AKAP79/150-AC interactions with respect to the AMPA receptor. Elegant studies by Hell and colleagues have suggested that ACs are recruited into larger signaling networks that include β-adrenergic receptors, kinases and ion channels (26,27). We have shown that AKAP79 functions to scaffold its cargo of signaling enzymes to synaptic AMPA receptors (9). Furthermore, GluR1 co-immunoprecipitates significant AC activity from rat brain extracts, in accord with previous reports of AC association with a GluR1 complex (27). We have shown that AKAP79 functions to scaffold its cargo of signaling enzymes to synaptic AMPA receptors (9). Therefore, it seemed possible that AKAP79 may also function to recruit AC5 into a similar type of multi-protein signaling network. Analysis of the AC5-AKAP79-AMPA receptor interactions were performed in two stages. Initial studies monitored changes in the co-purification of AC5 activity with the GluR1 subunit of the AMPA receptor. Rat brain extracts were incubated with the AKAP79-AKAP79 disruptor (AKAP79^{77-153}) or a control fragment (AKAP79^{109-290}). The GluR1 subunit of the AMPA channel was immunoprecipitated and co-purification of AC activity was measured upon stimulation with Gαs and forskolin. AKAP79^{77-153}
decreased GluR1-associated AC activity by an average of 49% (Fig. 7A), suggesting that AKAP150 mediates AC5/6 interactions with GluR1 in rat brain. In addition, disruption of AC5/6 association with the rat ortholog AKAP150 led to the reduction (56%) of AKAP150-associated AC activity (Fig. 7A), indicating that additional AC isoforms may be present in AKAP79/150 complexes.

The next phase of these studies examined this phenomena in the brains of AKAP null mice. Brain extracts were prepared from two AKAP knockout mouse strains: AKAP150 and WAVE-1, a neuronal anchoring protein that is concentrated in the dendrites (28,29). Initial characterization confirmed the ablation of each anchoring protein gene by immunoblotting (Fig 7B, top and middle panels). Loading controls indicated that equal amounts of protein were loaded in each lane (bottom panel). Immunohistochemical analysis further demonstrated the loss of AKAP150 expression in cultured hippocampal neurons when compared to wild type (Fig 7C).

To determine the extent to which AKAP79/150 is the sole AKAP that anchors AC to a GluR1 complex, we have repeated these experiments using brain tissue from wild type and AKAP150 null mice. AKAP150-associated AC activity is completely lost in brain from AKAP150 null mice but not in the knockout of another AKAP expressed in brain, WAVE-1 (Fig. 7D). As in rat, incubation with AKAP7977-153 led to a 50% loss in GluR1-associated AC activity, once again indicating the presence of AKAP79/150-anchored AC5/6. AC activity associated with GluR1 was also reduced by 70% in AKAP150 null tissue as compared to wild-type control (Fig. 7D). However, a significant amount of GluR1-associated AC activity (30%) remained in AKAP150 null tissue, suggesting some involvement of additional scaffolding proteins in anchoring AC to GluR1. Control experiments using WAVE-1 brains confirmed that this anchoring protein is not involved in anchoring AC to GluR1 as no loss in GluR1-associated AC activity is observed in WAVE-1 knockout tissue.

**DISCUSSION**

Organization of the cAMP synthesis machinery with PKA and its preferred substrates is believed to ensure the optimal relay of second messenger signals. AKAPs provide a molecular framework for the relay of this information by bringing together different components of these signaling pathways. Considerable information about how AKAPs function has been gleaned from analysis of the AKAP79/150 group of anchoring proteins (1). In this study we have employed the methods of protein chemistry and fluorescence microscopy to show that: 1) a subset of AC isoforms can directly interact with AKAP79; 2) this protein-protein interaction proceeds through the N-terminus of AC which docks with the second polybasic region of AKAP79; and 3) AKAP79/150 is responsible for linking AMPA receptors to AC5/6 activity in brain. Collectively, this information provides us with a deeper understanding of how an AKAP signaling complex is assembled.

**AKAP79 Selectivity for AC Isoforms**–In recent years AKAP79/150 has been shown to interact with a range of kinases, phosphatases, other scaffolding proteins and effector proteins. We first became interested in its role as an organizer of the cAMP synthesis machinery when we were able to co-purify AKAP79/150 with AC isoforms 5 and 6 from brain extracts (6). A logical extension of this work was to establish if other AC isoforms were capable of binding to AKAP79. As shown in figure 1, the AC 2, 3, 5, 6, 8 and 9 isoforms co-purify with AKAP79. This finding suggests that AC interaction with AKAP79 is a much more general phenomenon than we originally appreciated. However, it is worth noting that many of the associated AC isoforms are also regulated by one or more enzymes that interact with AKAP79. For example, PKA phosphorylates AC 5 and 6 to suppress cAMP synthesis, whereas PKC phosphorylates AC 2, 5 and 6 to modulate their activities (30-34). Likewise, CaM and protein phosphatase 2B are effectors of AC8 (35) and AC9 (36), respectively. This is reflected in the regulation of cAMP synthesis upon co-expression of AKAP79 of at least a subset of AC isoforms. Thus, the duration and magnitude of the cAMP may be dependant in part on which AC isoform is associated with the AKAP79 complex.

**Interaction Domains for AKAP79 and ACs**–Our data supports a model where multiple AC isoforms have the capacity to interact with AKAP79/150. This would imply that a common motif or structural fold on the AC provides a
determinant for AKAP binding. Structurally, C1 and C2 domains of all AC isoforms are highly conserved, whereas the N-terminal intracellular regions are highly variable. Our data suggest that the AKAP79 binding site is located in the variable regions of at least a subset of AC isoforms. Interactions of AC N-termini with AKAP79 would possibly explain differences in AKAP79 association/inhibition of ACs with similarities in sequence and regulatory patterns. Of the AC isoforms associated with AKAP79 in Fig. 1A, only AC 5, 6 and 9 utilize their N-termini as AKAP79 binding sites.

The three polybasic regions in the amino terminal regions of AKAP79/150 form the binding sites for PKC, CaM, F-actin, phosphatidylinositol 4,5-bisphosphate, and cadherin (4,11,12,14,37). Any two of these regions are sufficient to confer plasma membrane association and lateral targeting in epithelial membranes with E-cadherin (12,14). Although the PKC binding site has been further refined to the first polybasic region, additional mapping has not been performed for the other binding partners. Our mapping studies suggest that the second polybasic region of AKAP79 is required for binding to AC5 (Fig. 3). This region was confirmed as key for AC5-AKAP79 interactions since live cell imaging experiments demonstrate that deletion of residues 77-108 from AKAP79 abolishes its ability to interact with AC. This region only appears to encompass a binding site AC 5 and 6, since the disruptor AKAP79 77-153 cannot compete for binding with AC 2 or 9. It is unclear whether interactions with AC5/6 would alter association of AKAP79 with F-actin or cadherin. In addition, the question remains as to whether AC5/6 binding to AKAP79/150 will be subject to regulation by Ca²⁺/CaM or PKC phosphorylation. Finally, it is unknown whether AKAP79 retains the ability to interact with AC2 or AC9 when in complex with AC5. These latter AC isoforms clearly utilize different binding sites on AKAP79 that have yet to be defined. These findings suggest several AC binding sites may reside in AKAP79 and that additional factors such as tissue specific expression may contribute to the formation of distinct AC-AKAP complexes in vivo. An added complexity may be that other anchoring proteins such as Yotiao and mAKAP may compete for association with the available pool of ACs (7,8).
portion of AC activity must also be associated with GluR1 in an AKAP79/150- and WAVE-1-independent manner. These findings also imply that other AC isoforms may exist in complex with GluR1 or its binding partners. In the hippocampus this may reflect complexes with AC8 or AC9 (42,43).

**Summary**—We have demonstrated that AKAP79 can interact with a unique set of AC isoforms, playing either a passive scaffolding or inhibitory role. We have also identified a unique disrupting agent, specific for AC5/6-AKAP79 that we used to determine the composition of GluR1 complexes in brain. The regulation of GluR1 by AKAP79/150 is clearly important for synaptic transmission and memory retention (23,44-46). The association to these complexes of calcium-stimulated or calcium-inhibited AC isoforms will set up important feedback or feed-forward pathways to control GluR1 regulation in specific regions of brain.

**REFERENCES**


**FOOTNOTES**

The authors thank Kathryn Hassell for excellent technical assistance, Dr. Mark Dell’Acqua for PKA-RII-YFP and AKAP79ΔPKA-CFP, and Dr. James Broughman, Cytodynamic Imaging Facility for help with microscopy applications.

This work was supported by NIH grants GM60419 to CWD and GM48231 to JDS.

The abbreviations used are: AC, adenylyl cyclase; AKAP, A-Kinase Anchoring Protein; AMPA, α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate; CaM, calmodulin; CFP, cyan fluorescence protein; FLIM, fluorescence lifetime imaging microscopy; FRET, fluorescence resonance energy transfer; Fsk, forskolin; GFP, green fluorescence protein; GluR1, AMPA receptor type 1 subunit; GST, glutathione S-transferase; HEK, Human embryonic kidney; NKA, Na⁺,K⁺-ATPase; NMDA, N-methyl-D-aspartic acid; NT, N-terminus; PKA, protein kinase A; PKC, protein kinase C; YFP, yellow fluorescence protein.

**FIGURE LEGENDS**

**FIG. 1.** AC types 2, 3, 5, 6, 8 and 9 associate with AKAP79. **A**, HEK293 cells were transfected with AC isoforms 1 through 9 +/- AKAP79-FLAG. Samples were immunoprecipitated with anti-FLAG agarose and assayed for AC activity with 50 nM Gαs and 100 µM forskolin. Samples transfected with AKAP79 plus AC 2, 3, 5, or 6 (p<0.05) had significant associated Gαs/forskolin-stimulated AC activity compared to the AC isoform alone. AKAP79 expression was confirmed by Western blotting (Sup. Fig. 1). **B**, IP-AC assay of AKAP79 and AC9 as performed in A, using 200 nM Gαs to stimulate AC9. Samples transfected with AKAP79 plus AC9 (p<0.05) had significant associated Gαs-stimulated AC activity compared to AC9 alone. **C**, AC8 association with AKAP79 was detected when immunoprecipitates were assayed with 100 µM Ca²⁺ and 300 nM CaM (p<0.05).

**FIG. 2.** AKAP79 inhibits AC 2, 5 and 6. Membranes from HEK293 cells transfected with vector or AC 2, 3, 5, 6 and 9 +/- AKAP79 were stimulated with 50 nM Gαs (A) or 200 nM Gαs for AC9 (B). AC 2, 5 and 6, but not AC 3 or 9, were inhibited in the presence of AKAP79 (p<0.05).

**FIG. 3.** Mapping of AC N-termini binding domains on AKAP79. **A**, GST-tagged AC N-termini were screened for interactions with AKAP79 by GST-pull down using HEK293 cell lysates expressing AKAP79. AKAP79 bound the N-termini of AC 5, 6 and 9, but not AC 2 or 3. **B**, Schematic diagram of AKAP79 and deletion constructs used in panels C and D. Dotted lines indicate the interaction domain for AC5-NT. **C**, GST pull-down assay was performed using GST or GST-AC5-NT incubated with HEK293 cell lysates expressing vector or AKAP79 Myc-tagged fragments. **D**, Purified AKAP79 S-tagged fragments 77-153 and 109-290 were pulled-down with GST, GST-AC5-NT or GST-AC5Δ60-NT. S-tag Western blot shows direct binding of AC5-NT with AKAP7977-153.

**FIG. 4.** AKAP79⁷⁷-¹⁵³ specifically competes for binding and inhibition of AC5/6-AKAP79 complexes. **A**, An IP-AC assay was performed as indicated in Fig. 1A in the presence of 10 µM AKAP79⁷⁷-¹⁵³ or control polypeptide AKAP79¹⁰⁹-²⁹⁰. Addition of AKAP79⁷⁷-¹⁵³ disrupts associations between AKAP79 and AC 5 and 6, but not AC 2 or 9. **B**, Membranes from HEK293 cells expressing AC5 +/- AKAP79 were incubated with the indicated concentrations of purified AKAP79⁷⁷-¹⁵³ prior to stimulation with 50
nM Gαs + 100 μM forskolin. AKAP7977-153 reverses the inhibition of AC5 by AKAP79 in a concentration-dependent manner. C, Dose-response curve from B indicates an IC₅₀ of 1.0 μM for the reversal of AKAP79 inhibition by AKAP7977-153.

**FIG. 5.** Cellular interaction of AKAP79-CFP and AC5-YFP in HEK293 cells by intensity-based FRET. A, Characterization of fluorescent protein-tagged proteins. Membranes from HEK293 cells expressing AKAP79 and AC5 (with and without fluorescent protein tags), were stimulated with 50 nM Gαs + 100 μM forskolin. Fluorescent protein tags did not affect AC activity and its inhibition by AKAP79. B, FRET analysis of AKAP79 and AC5 in HEK293 cells. Fluorescence microscopy images of HEK293 cells transiently transfected with the indicated proteins were recorded using three different channels: 1) donor, CFPex/CFPem; 2) FRET, CFPex/YFPem; and 3) acceptor, YFPex/YFPem. A representative cell is shown for each set (top, AKAP79-CFP and NKAα1-YFP; middle, AKAP79-CFP and AC5-YFP; bottom, AKAP79ΔB-CFP and AC5-YFP). Pseudocolor FRET/C/CFP images were obtained using Slidebook software. Scale bar, 10 μm. C, Quantitative analysis of FRET by N₉FRET method (n = 4, using images from 16 to 22 cells, p<0.01).

**FIG. 6.** FLIM-FRET of AKAP79-CFP and AC5-YFP. A, HEK293 cells expressing AKAP79-CFP alone or with PKA RII-YFP or AC5-YFP were imaged in the frequency domain in a wide-field FLIM microscope. The lifetime of AKAP79-CFP is significantly decreased when co-expressed with PKA RII-YFP or AC5-YFP, as a result of FRET between CFP and YFP. B, The lifetime of AKAP79ΔB-CFP does not change when co-expressed with AC5-YFP. Graphical quantitation of CFP-tagged protein lifetimes is shown for each panel (mean ± S.E.M.; n = 3; 60-120 cells).

**FIG. 7.** GluR1 associates with AC5/6 in brain in an AKAP150-dependent manner. A, Rat brain extracts were preincubated with either 77-153 or 109-290 AKAP79 polypeptides prior to immunoprecipitation using IgG (control), or antibodies against GluR1 or AKAP150. Immunoprecipitates were stimulated with Gαs/forskolin and associated AC activity was measured (n=3). B, Western blot analysis of brain extracts from wild-type, AKAP150⁺⁰⁺, or WAVE-1⁻⁻ mice are shown. C, Immunohistochemistry of hippocampal neurons from wild-type or AKAP150⁻⁻ mice indicates the loss of AKAP150. D, IP-AC assays were performed as in A, using brain tissue from wild-type, AKAP150, or WAVE-1 null mice. All samples include the control polypeptide AKAP79109-290, except where AKAP7977-153 is indicated by $ (n=3).
Figure 1
Figure 2

A

B

![Graph showing AC activity](image)

**Figure 2**

*Note: The figure shows the effect of different conditions on AC activity. The graphs indicate the presence of AKAP79 and its interaction with Gαs, as well as the effect of various AC isoforms (AC2, AC3, AC5, AC6) and pcDNA3 transfection.*
Figure 3

A

GST-AC-NT

Input

A79

GST 2 3 5 6 9

α-GST

30 kD

B

AKAP79 full length

Polybasic regions

PKC

PKA

PP2B

1

A B C

31-52 77-101 116-145

315-360 392-413

153

153

154

427

109

290

C

AKAP79

pc3 1-153 154-427 77-153

+ + + + +

GST GST-AC5NT

1-153 77-153

154-427

D

AKAP79 fragments

77-153 109-290

-5NT 5NT

-5NT 5NT

α60 α60

77-153 77-153

109-290

GST
Figure 4

A

G<sub>αs/Fsk</sub>

AC activity (pmol/IP)

AKAP79 77-153, µM

0 2468 1 0 50 60 70 80 90 100

+ + + + + + + + + +

AC2 AC5 AC6 AC9

B

G<sub>αs/Fsk</sub>

AC activity (nmol/mg/min)

AKAP79 77-153, µM

0 1 3 5 10

+ + + + +

C

G<sub>αs/Fsk</sub>

AC activity (% Control)

AKAP79 77-153, µM

0 2 4 6 8 10

* * * * *
Figure 5

A79-CFP   NKA
1-YFP      Merged    FRETC/CFP
A79-CFP      AC5-YFP       Merged     FRETC/CFP
A79-B-CFP     AC5-YFP      Merged    FRETC/CFP

AC activity (nmol/mg/min)

0 1 2 3 4 5

A79 AC5 AC5 +A79
A79 AC5 AC5 +A79
Non-tagged       CFP- and YFP-tagged

0.0 0.5 1.0 1.5

N_{FRET}

Acceptor: NKA AC5 AC5 RII RII
Donor: A79 A79 A79 A79 ΔB ΔPKA

B

A79-CFP  NKAα1-YFP  Merged  FRET^C/CFP

A79-CFP  AC5-YFP  Merged  FRET^C/CFP

A79ΔB-CFP  AC5-YFP  Merged  FRET^C/CFP

Downloaded from http://www.jbc.org/ by guest on May 3, 2016
Figure 6

**A**

PKC

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td></td>
<td>427</td>
</tr>
</tbody>
</table>

Lifetime (ns)

- A79-CFP
- A79-CFP + RII-YFP
- A79-CFP + AC5-YFP

**B**

PKC

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td>77-108</td>
<td></td>
</tr>
</tbody>
</table>

Lifetime (ns)

- A79ΔB-CFP
- A79ΔB-CFP + AC5-YFP

**Graph**

- Lifetime (ns) for A79-CFP
- Lifetime (ns) for A79ΔB-CFP

**Legend**

- PKA
- PP2B

**X-axis**

- AC5-YFP
- RII-YFP

**Y-axis**

- Lifetime (ns)
Figure 7

A

B

C

D

AC activity (norm. to IgG)

0 1 2 3 4 5

109-290 77-153

IgG αGluR1 αA150

++ ++

αAKAP150 αGluR1

WT KO

αAKAP150

α-WAVE

α-tubulin

250 200 150 100 50

75 50 25 12.5

WT A150 KO Wave KO

AC activity (pmol/IP)

0.0 0.2 0.4 0.6 0.8 1.0

IgG αAKAP150 αGluR1

+ + + + + +

α-WAVE αGluR1

α-tubulin

WT A150 KO Wave KO

by guest on May 3, 2016 http://www.jbc.org/ Downloaded from
SUPPLEMENTAL DATA

Supplemental Table 1. **Quantitation of FRET by three different methods.** Values are given as mean ± S.E.M.; *, p<0.05 versus AKAP79 and NKAα1 control; $, p<0.05 versus the corresponding deletion (AC5 and AKAP79ΔB or PKA RII and AKAP79ΔPKA).

Supplemental Figure 1. **Total AC activity associated with overexpression of AC isoforms 1-9.** HEK293 cells were transfected with AC isoforms 1 through 9 +/- AKAP79-FLAG. Samples were immunoprecipitated with anti-FLAG agarose. The starting extracts (Sup. Fig. 1) and immunoprecipitations (shown in Fig. 1A) were assayed for AC activity stimulated with 50 nM Gα302s and 100 μM forskolin (A) or 200 nM Gαs (B). The overexpression of each AC isoform was confirmed by the increase in AC activity compared to vector alone. AKAP79 expression was confirmed by Western blotting.

Supplemental Figure 2. **FLIM-FRET of AKAP79ΔPKA-CFP and PKA RII-YFP.** HEK293 cells expressing AKAP79ΔPKA-CFP alone or with PKA RII-YFP were imaged in the frequency domain in a wide-field FLIM microscope. Deletion of the PKA binding domain (AKAP79ΔPKA-CFP) abolishes a change in lifetime when co-expressed with PKA RII-YFP. Graphical quantitation of CFP-tagged protein lifetimes is shown (mean ± S.E.M.; n = 3; 60-120 cells).
## Supplemental Table 1

<table>
<thead>
<tr>
<th>Donor Protein</th>
<th>Acceptor Protein</th>
<th>$FRET^N$</th>
<th>$N_{FRET}$</th>
<th>$FRET^C/I_{CFP}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>AKAP79</td>
<td>NKAα1</td>
<td>0.028 ± 0.005</td>
<td>0.430 ± 0.023</td>
<td>0.788 ± 0.112</td>
</tr>
<tr>
<td>AKAP79</td>
<td>AC5</td>
<td>0.067 ± 0.012*</td>
<td>1.003 ± 0.102*</td>
<td>1.812 ± 0.283*</td>
</tr>
<tr>
<td>AKAP79ΔB</td>
<td>AC5</td>
<td>0.037 ± 0.006</td>
<td>0.576 ± 0.053*</td>
<td>1.013 ± 0.124</td>
</tr>
<tr>
<td>AKAP79</td>
<td>PKA RII</td>
<td>0.076 ± 0.012*</td>
<td>1.179 ± 0.087*</td>
<td>2.092 ± 0.250*</td>
</tr>
<tr>
<td>AKAP79ΔPKA</td>
<td>PKA RII</td>
<td>0.034 ± 0.005</td>
<td>0.521 ± 0.032*</td>
<td>0.932 ± 0.107</td>
</tr>
</tbody>
</table>

*Significant difference compared to control.
Supplemental Figure 1

A

G_{\alpha}\text{Fsk}

\begin{align*}
\text{AC activity (pmol)}
\end{align*}

AKAP79 - + - + - + - + - + - + - + - + - + - + - +

pc3 AC1 AC2 AC3 AC4 AC5 AC6 AC7 AC8

B

G_{\alpha}

\begin{align*}
\text{AC activity (pmol)}
\end{align*}

AKAP79 - + - + pc3 AC9

C

Ca^{2+}/CaM

\begin{align*}
\text{AC activity (pmol)}
\end{align*}

AKAP79 - + - + pc3 AC8
Supplemental Figure 2

The graph shows the lifetime (ns) of AKAP79ΔPKA-CFP and AKAP79ΔPKA-CFP + PKA-RII-YFP. The chart on the right illustrates the lifetime (ns) of PKA RII-YFP with and without A79ΔPKA-CFP.
AKAP79 interacts with multiple adenylyl cyclase (AC) isoforms and scaffolds AC 5 and 6 to AMPA receptors
Riad Efendiev, Bret K. Samelson, Bao T. Nguyen, Prasad V. Phatarpekar, Faiza Baameur, John D. Scott and Carmen W. Dessauer

J. Biol. Chem. published online March 15, 2010

Access the most updated version of this article at doi: 10.1074/jbc.M110.109769

Alerts:
  • When this article is cited
  • When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

Supplemental material:
http://www.jbc.org/content/suppl/2010/03/15/M110.109769.DC1.html

This article cites 0 references, 0 of which can be accessed free at
http://www.jbc.org/content/early/2010/03/15/jbc.M110.109769.full.html#ref-list-1