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Targeting of PKA to Glutamate Receptors through a MAGUK-AKAP Complex

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Summary

Compartmentalization of glutamate receptors with the signaling enzymes that regulate their activity supports synaptic transmission. Two classes of binding proteins organize these complexes: the MAGUK proteins that cluster glutamate receptors and AKAPs that anchor kinases and phosphatases. In this report, we demonstrate that glutamate receptors and PKA are recruited into a macromolecular signaling complex through direct interaction between the MAGUK proteins, PSD-95 and SAP97, and AKAP79/150. The SH3 and GK regions of the MAGUKs mediate binding to the AKAP. Cell-based studies indicate that phosphorylation of AMPA receptors is enhanced by a SAP97-AKAP79 complex that directs PKA to GluR1 via a PDZ domain interaction. As AMPA receptor phosphorylation is implicated in regulating synaptic plasticity, these data suggest that a MAGUK-AKAP complex may be centrally involved.

Introduction

In the mammalian central nervous system, the majority of fast excitatory transmission is mediated by ionotropic glutamate receptors. Like most neurotransmitter receptors, glutamate receptors are subject to modulation by protein phosphorylation (Swope et al., 1999). Regulation of glutamate receptor function by protein kinases and phosphatases is particularly interesting given their central role in various forms of synaptic plasticity. A great deal of evidence suggests that kinases and phosphatases are critically involved in the induction and maintenance of long-term potentiation (LTP) and long-term depression (LTD), two well-studied cellular models for learning and memory (recently reviewed by Malenka and Nicoll, 1999). Intense investigation has focused on understanding the molecular mechanisms underlying these phenomena.

In recent years, a host of proteins comprising the postsynaptic density (PSD), the electron dense cytoskeletal specialization of excitatory synapses, has been identified and characterized. Prominent among these synaptic organizing molecules are a growing number of proteins that contain interaction modules called PDZ domains (named for PSD-95, Discs large, and ZO-1). PDZ proteins have been implicated in the targeting and clustering of ion channels, including glutamate receptors at synaptic sites (Kennedy, 1997; Sheng and Wyszynski, 1997; Ziff, 1997; O'Brien et al., 1998). PDZ proteins are also likely to regulate signal transduction events by recruiting additional signaling molecules to receptors.

Certainly the best studied family of PDZ proteins is the membrane-associated guanylate kinases (MAGUKs), of which PSD-95 (SAP90) is the prototypic member (Anderson, 1996). The MAGUKs are modular proteins composed of three N-terminal PDZ domains, followed by a src homology 3 (SH3) domain and a guanylate kinase–like (GK) region. The C-terminal cytoplasmic tail of NMDA receptor subunits contains a canonical PDZ recognition motif (S/T-X-I/V) that mediates high-affinity binding to the PDZ domains of PSD-95 and other MAGUK family members (Kornau et al., 1995; Kim et al., 1996; Lau et al., 1996; Muller et al., 1996). When coexpressed in heterologous cells, PSD-95 and NMDA receptors form surface clusters, suggesting that PSD-95 may function to cluster NMDA receptors at synaptic sites in vivo (Kim et al., 1996). Curiously, in PSD-95 mutant mice, NMDA receptor localization at the synapse is normal (Migaud et al., 1998). The mice do, however, exhibit defects in LTP, LTD, and spatial learning, suggesting that PSD-95 plays a critical role in coupling NMDA receptors to downstream signaling events.

A distinct set of PDZ proteins interact with AMPA receptors. GRIP1/2 (glutamate receptor interacting proteins 1 and 2; Dong et al., 1997, 1999; Wyszynski et al., 1999), ABP (AMPA receptor binding protein; Srivastava et al., 1998), and Pick-1 (protein interacting with C kinase 1; Xia et al., 1999) bind to, via their PDZ domains, the C-terminal tails of GluR2/3 subunits of AMPA receptors. Dominant-negative experiments suggest that these interactions are important for the synaptic clustering of AMPA receptors (Dong et al., 1997). In addition, the MAGUK family member SAP97 associates with the GluR1 subunit of AMPA receptors via a PDZ-C-terminal (TGL) interaction (Leonard et al., 1998). Thus, multiple PDZ proteins may act in concert to coordinate the localization of AMPA receptors at synapses.

The details of how protein kinases and phosphatases are targeted to specific synaptic substrates are less well understood. Early electrophysiological studies provided evidence that targeting of protein kinase A (PKA) is required for modulation of AMPA receptors in hippocampal neurons (Rosenmund et al., 1994). At the molecular level, PKA targeting is achieved through A kinase anchoring proteins (AKAPs), proteins that bind to the regulatory (R) subunit dimer of PKA to direct the holoenzyme to discrete subcellular structures (Colledge and Scott, 1999). AKAP79/150, which provides a scaffold for three signaling enzymes—PKA, protein kinase C (PKC), and the calcium-dependent phosphatase calcineurin—has been a leading candidate in the regulation of ion channel activity (Carr et al., 1992; Coghlan et al., 1995). It has been postulated that AKAP79/150 functions to target its associated enzymes to ion channels, such as AMPA

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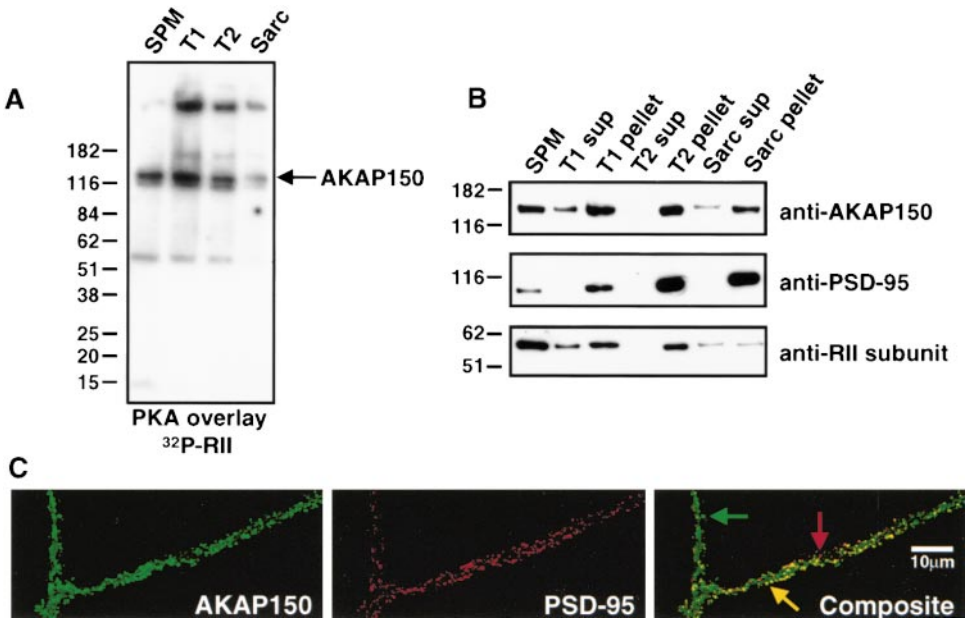

Figure 1. AKAP150 Is Present in the PSD and at Synaptic Sites in Cultured Hippocampal Neurons

(A) AKAP150 is a major PKA binding protein in PSD fractions. Synaptic plasma membranes (SPM) were extracted with Triton X-100 once (T1), twice (T2), or with Triton followed by sarcosyl (Sarc). A sample (2 μg) of each insoluble PSD fraction was separated by SDS–PAGE and transferred to nitrocellulose membrane. PKA binding proteins were identified by incubating membranes with ^{32}P-labeled regulatory II subunit of PKA (RII). RII binds to AKAP150. The higher molecular weight RII binding protein may correspond to MAP2, a previously identified AKAP. RII also homodimerizes with the PKA R subunit (55 kDa).

(B) Fractionation of AKAP150, RII, and PSD-95 in PSDs. Various soluble (sup) and insoluble (pellet) PSD fractions (1 μg each) were immunoblotted with antibodies specific for AKAP150 (top), PSD-95 (middle), and RII (bottom). AKAP150 and RII are present in the core PSD (Sarc pellet), along with PSD-95.

(C) Distribution of AKAP150 in hippocampal neurons. Three-week-old cultured hippocampal neurons were labeled simultaneously with antibodies against AKAP150 (left) and PSD-95 (middle). AKAP150 exhibits a punctate pattern of distribution along dendrites that codistributes with PSD-95 labeling at a majority of PSD-95-positive synaptic sites. Representative regions are highlighted that contain both proteins (yellow arrow), AKAP150 alone (green arrow), and PSD-95 alone (red arrow).

receptors, where they are optimally positioned to alter the phosphorylation state of the channel (Rosenmund et al., 1994; Colledge and Scott, 1999). In this report, we provide evidence that the AKAP79/150 signaling scaffold is recruited to glutamate receptors via direct association with MAGUK proteins. Cell-based studies suggest that AKAP79 is targeted to NMDA receptors through interaction with PSD-95. In the case of AMPA receptors, SAP97 links AKAP79 to GluR1. These protein–protein interactions position PKA near the receptor, permitting specific phosphorylation of serine 845, a regulatory site within the cytoplasmic tail of GluR1.

Results

AKAP150 Is a Major PKA Binding Protein in the PSD

To characterize AKAP signaling complexes at the synapse, we used an RII overlay assay (Hausken et al., 1998) to identify AKAPs in PSD fractions (Figure 1A). Rat brain PSDs were purified by sequential extraction of synaptic plasma membranes with Triton X-100 and sarcosyl detergents (Cho et al., 1992). We consistently observed a predominant RII binding protein that migrated at ~150 kDa in each PSD fraction, including the core fraction (Sarc). A higher molecular weight binding protein that may correspond to MAP2, a previously identified AKAP, was often observed (Vallee et al., 1981; Rubino et al., 1989). The 55 kDa R subunit of PKA itself also binds to RII in this assay, as R subunits form homodimers (Hausken et al., 1994). Given its apparent molecular weight, we predicted that the 150 kDa RII binding protein was AKAP150, the rat ortholog of human AKAP79. Despite the discrepancy in size, AKAP79 and AKAP150 are highly conserved and appear to be functional orthologs; the latter contains a central repeat sequence of unknown function that accounts for its higher molecular weight.

To confirm that the RII binding protein was indeed AKAP150, we studied the fractionation pattern of AKAP150 in PSDs by immunoblotting (Figure 1B). AKAP150 showed the same pattern of distribution in the PSDs as the 150 kDa RII binding protein. It was highly enriched in the insoluble PSD fractions—the majority was resistant to extraction by Triton X-100 and sarcosyl detergents (Figure 1B, top). In fact, a significant amount of AKAP150 was found along with PSD-95, a well-defined PSD protein (Figure 1B, middle), in the core PSD fraction (Sarc pellet). In addition, RII showed a similar extraction pattern to AKAP150 (Figure 1B, bottom). Together, these data suggest that AKAP150 is a major PKA binding protein in the PSD.

Previous work indicated that AKAP150 is distributed throughout the dendritic tree in cultured hippocampal neurons (Glantz et al., 1992; Dell'Acqua et al., 1998). To determine whether AKAP150 was specifically localized

at synaptic sites, we examined the distribution of AKAP150 relative to PSD-95, a marker for excitatory glutamatergic postsynaptic sites. Cultured hippocampal neurons were labeled simultaneously with antibodies against AKAP150 and PSD-95 (Figure 1C). AKAP150 showed a punctate pattern of distribution along dendrites that overlapped with PSD-95 labeling. AKAP150 codistributed with PSD-95 at a majority of PSD-95 positive synaptic sites. This is best observed in the composite image (Figure 1C, right). Collectively, these data indicate that AKAP150 and PSD-95 are colocalized in a subset of excitatory synapses, a finding consistent with the subcellular distribution data.

AKAP150 Associates with PSD-95 Family of Proteins In Vivo

On the basis of our results that AKAP150 and PSD-95 are found together in the core PSD fraction and at synaptic sites in neurons, we wondered whether both proteins might be present in the same complex. To test this idea, we isolated R subunit-AKAP complexes from rat brain extracts by affinity chromatography on cAMP agarose and looked for copurifying PSD-95 (Figure 2A). Indeed, PSD-95 was eluted from cAMP agarose (Figure 2B, left). In control conditions, in which excess cAMP was added to extracts to block specific binding to the beads, PSD-95 was absent from the eluants (Figure 2B, left). These data suggest that PSD-95 specifically associates with PKA-R subunit complexes in brain. Furthermore, using a pan antibody that recognizes multiple MAGUK family members, including PSD-95, chapsyn110/PSD-93, and SAP97 (Upstate Biotechnology, catalog number 05–427), we consistently observed two higher molecular weight proteins, in addition to PSD-95, that were specifically eluted by cAMP (Figure 2B, right). These results suggest that multiple MAGUK family members form complexes with PKA in brain.

To rule out the possibility that PSD-95 was itself an AKAP, we tested for direct interaction with the RII subunit by a variety of solid phase and solution binding assays. We found no evidence for direct association between PSD-95 and RII (data not shown). This suggested that an intermediate protein, such as AKAP150, is required. To test this hypothesis, we performed in vitro GST pulldown experiments with purified proteins (Figure 2C). (Since the full-length cDNA of AKAP150 has not been cloned, we have used AKAP79 cDNA constructs for in vitro binding and heterologous expression experiments.) Beads charged with various GST fusion proteins (Figure 2C, bottom) were incubated with recombinant purified AKAP79. AKAP79 bound to both GST-PSD-95 and GST-SAP97, but not to control GST. In addition, AKAP79 was not retained by GST-Grb2, another adaptor protein, which like the MAGUK proteins contains an SH3 domain, nor by GST-Rab, an unrelated protein (Figure 2C, top). These data indicate that AKAP79 interacts directly with PSD-95 and SAP97. The binding appears specific for MAGUK proteins, as AKAP79 does not bind to other GST fusion proteins.

To verify that PSD-95 and AKAP150 form a complex in vivo, we performed coimmunoprecipitation experiments from deoxycholate solubilized rat synaptosomal membranes (Figure 2D). PSD-95 antibodies immunoprecipitated AKAP150 from extracts (Figure 2D, top left), while control IgG antibodies did not. In addition, WAVE, another AKAP expressed in brain (R. S. Westphal and J. D. S., unpublished data), was not found in PSD-95 immunoprecipitates (Figure 2D, bottom left). In reciprocal experiments, AKAP150 antibodies immunoprecipitated PSD-95 (Figure 2D, top right). Control immunoprecipitations with yotiao (a brain AKAP associated with NMDA receptors; Westphal et al., 1999) antibodies did not contain PSD-95. Furthermore, synaptophysin, an unrelated synaptic protein, was not found in AKAP150 complexes (Figure 2D, bottom right). These data provide strong evidence for a specific interaction between PSD-95 and AKAP150 in synaptic fractions. Together, the data presented in Figure 2 suggest that AKAP150 directly interacts with MAGUK family members in brain.

PSD-95 and SAP97 Associate with AKAP150-Bound Kinases

Previous studies have shown that AKAP79/150 provides a scaffold for three second messenger–regulated signaling enzymes—PKA, PKC, and calcineurin (Coghlan et al., 1995; Klauck et al., 1996). We used GST pulldown assays to test whether PSD-95 and SAP97 associate with these signaling enzymes in brain extracts through their interaction with AKAP150 (Figure 3A). In addition to PSD-95, we also focused on SAP97 as this MAGUK family member recently was shown to associate with AMPA receptors (Leonard et al., 1998), known targets of PKA (Rosenmund et al., 1994; Roche et al., 1996). As expected, AKAP150 was isolated from extracts by both GST-PSD-95 and GST-SAP97, but not by control GST beads (Figure 3B, first [top] panel). Likewise, PKCα (Figure 3B, second panel) and the regulatory (RII) subunit of PKA (Figure 3B, third panel) also were specifically retained by PSD-95 and SAP97. Neither kinase was detected in control experiments using GST alone. Surprisingly, we were unable to isolate the phosphatase calcineurin in these experiments (Figure 3B, fourth [bottom] panel). These data suggest that signaling complexes containing PSD-95/SAP97, AKAP150, and its associated kinases, PKA and PKC, exist in brain. It is likely that other PKC isoforms also may be present in this signaling complex, as previous work has shown that AKAP150 can associate with multiple PKC isoforms in brain (Faux et al., 1999).

Although AKAP79 is known to directly interact with calcineurin (Coghlan et al., 1995; Kashishian et al., 1998), we were unable to isolate complexes containing PSD-95, AKAP79, and calcineurin in a variety of assays (data not shown). This suggested that binding of PSD-95 and calcineurin to AKAP79/150 might be mutually exclusive. To test this hypothesis directly, we performed a competition assay using recombinant proteins (Figure 3C). GST-PSD-95 beads were incubated with purified recombinant AKAP79 in the presence of increasing concentrations of purified calcineurin. In the absence of calcineurin, PSD-95 beads specifically retained AKAP79, while control GST beads did not. Calcineurin competed for binding of AKAP79 to PSD-95 in a dose-dependent manner; binding of AKAP79 to PSD-95 was decreased by 100 nM calcineurin and almost completely abolished by 200 nM (Figure 3C). These data suggest that PSD-95 competes with calcineurin for binding to AKAP79.

AKAP150 Binds to the SH3 and GK Domains of PSD-95

The PSD-95 family of MAGUK proteins are modular scaffolding proteins composed of several protein interaction

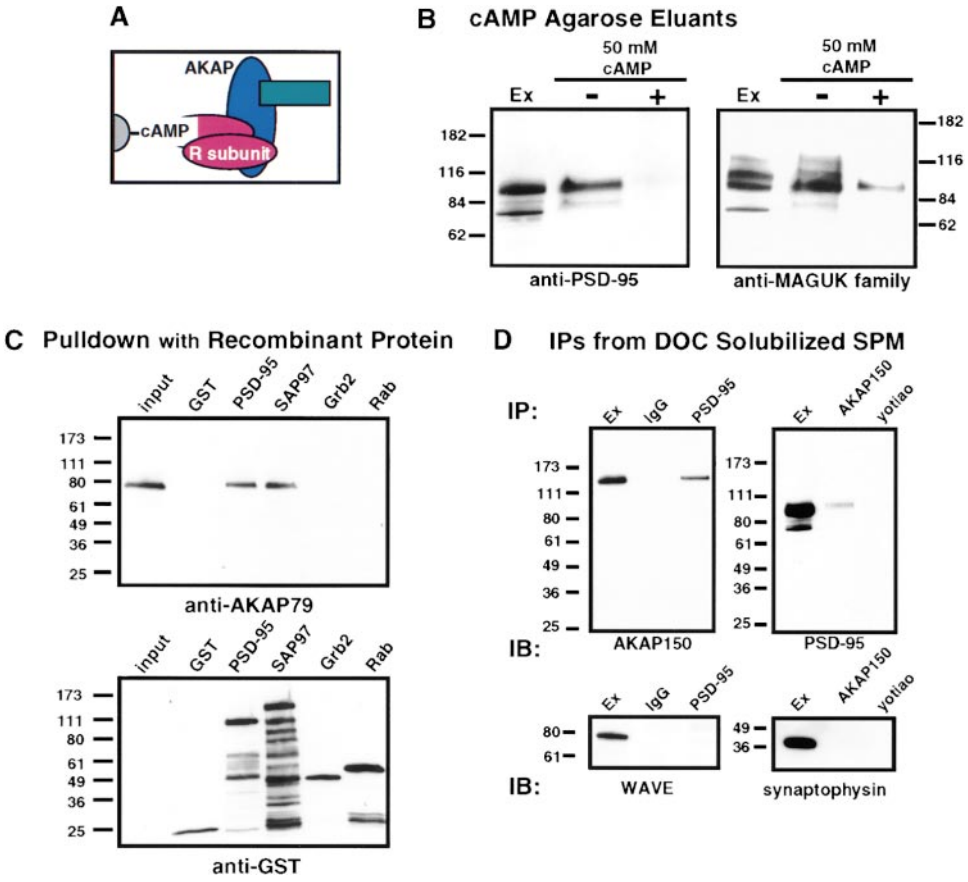

Figure 2. AKAP150 Associates with PSD-95 in Brain

(A) Schematic diagram showing purification of R subunit-AKAP complexes from brain extracts using cAMP agarose.

(B) Association of PSD-95 and family members with AKAP complexes in brain. Extracts from whole rat brain were incubated with cAMP agarose in the presence or absence of excess cAMP (50 mM). After extensive washing, bound proteins were eluted from beads with 75 mM cAMP and TCA precipitated. Pellets were resuspended in SDS sample buffer. Immunoblotting the eluants for PSD-95 shows that PSD-95 specifically bound to cAMP agarose (left). Immunoblotting with a pan-MAGUK antibody indicates that several family members bound to cAMP agarose (right).

(C) PSD-95 and SAP97 directly associate with AKAP79. A series of GST fusion protein beads were incubated with recombinant purified AKAP79 (0.5 μg), the human ortholog of AKAP150. Bound protein was eluted with SDS sample buffer and analyzed by immunoblotting with AKAP79 antibodies. The amount of AKAP79 used in each pulldown (0.5 μg) was run in the input lane. A large fraction of AKAP79 was retained specifically by PSD-95 and SAP97 beads. Control GST, Grb2, or Rab beads did not retain AKAP79 (top). The blot was stripped and reprobed with GST antibodies to show that equivalent amounts of each fusion protein were used in the pulldowns (bottom).

(D) Coimmunoprecipitation of PSD-95 and AKAP150 from brain. Extracts of rat forebrain synaptic plasma membranes (SPM) were immunoprecipitated with control nonimmune IgG, PSD-95, AKAP150, and yotiao antibodies. A fraction of the total protein used (1%) was run in the extract lane (Ex). AKAP150 specifically coimmunoprecipitated with PSD-95, but not control IgG antibodies (top left). WAVE, another AKAP expressed in brain, was not detected in PSD-95 precipitates (bottom left). In reciprocal experiments, PSD-95 coimmunoprecipitated with AKAP150, but not with yotiao, another brain AKAP (top right). Synaptophysin, a protein enriched in synaptic fractions, was not detected in AKAP150 precipitates (bottom right).

regions—three PDZ repeats, an SH3 domain, and a GK region. To identify which region of PSD-95 interacts with the AKAP, a family of GST fusion proteins encompassing the individual domains of PSD-95 were screened for their ability to isolate AKAP150 from rat brain extracts (Figures 4A and 4C). Given that the C terminus of AKAP150 lacks the canonical PDZ recognition motif, we predicted that the PDZ domains of PSD-95 would not bind to AKAP150. Indeed, neither PDZ1, PDZ2, nor PDZ3 bound to AKAP150 (Figure 4B). Surprisingly, both the SH3 and GK domains independently isolated AKAP150 from brain extracts (Figure 4B). Similar results were obtained using recombinant purified AKAP79 (data not shown). In addition, the SH3 and GK domains of SAP97 isolated AKAP150 from brain extracts (data not shown). These data suggest that MAGUK proteins interact with AKAP79/150 via two regions in their C terminus.

SH3 domains typically bind to proline-rich sequences containing a PXXP motif (Pawson and Scott, 1997). However, AKAP79/150 does not contain this conserved motif. To further characterize this interaction, we tested the ability of two SH3 domain mutants to interact with recombinant AKAP79 in GST pulldowns (Figures 4D). Protein staining of the membrane confirmed that equal amounts of wild-type and mutant SH3 domains were used in each pulldown (Figure 4E). It has been reported that mutation of tryptophan 470 to alanine (W470A) in the SH3 domain of PSD-95 disrupts SH3-proline peptide

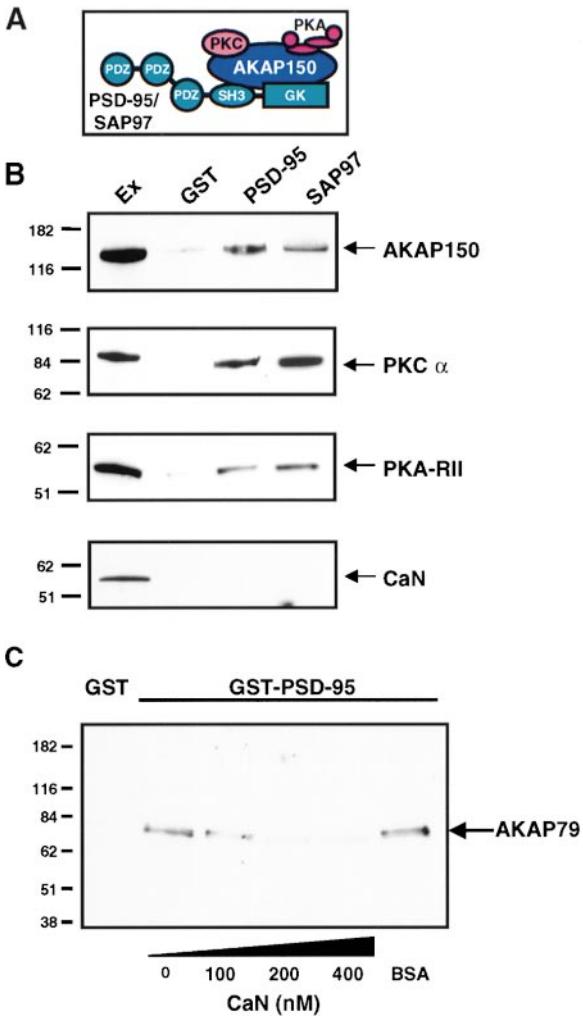

Figure 3. PSD-95 and SAP97 Interact with AKAP150-Associated Kinases

(A) Schematic diagram of proposed MAGUK-AKAP signaling complex.

(B) GST pulldown assays showing interaction of PSD-95 and SAP97 with AKAP150-associated kinases. GST, GST-PSD-95, and GST-SAP97 beads were incubated with whole rat brain extract. Bound proteins were analyzed by immunoblotting with the antibodies indicated. A sample of extract (5 μg) was run in the extract lane (Ex) to show that bound proteins migrate at the appropriate size. AKAP150 (first [top] panel), PKCα (second panel), and PKA-RII subunit (third panel) were specifically retained by PSD-95 and SAP97 but not control GST beads. The phosphatase calcineurin (CaN) was not detected in either PSD-95 or SAP97 pulldowns (fourth [bottom] panel). The migration position of each protein is indicated by an arrow.

(C) The ability of calcineurin to compete with PSD-95 for binding to AKAP79 in vitro was tested by competition assay. Purified recombinant AKAP79 (0.2 μg/11 nM) was incubated with equivalent amounts of control GST or GST-PSD-95 beads in the presence of increasing concentrations of purified calcineurin A (0, 100, 200, or 400 nM) or control BSA (400 nM). The amount of AKAP79 bound was analyzed by immunoblotting eluted samples with AKAP79 antibodies.

interactions without compromising SH3 domain structure (Erpel et al., 1995; McGee and Bredt, 1999). This mutation had no qualitative effect on the interaction with AKAP79 when compared to the wild-type SH3 domain (Figure 4D). In contrast, mutation of leucine 460 to proline (L460P) in the SH3 domain of PSD-95 greatly diminished binding to AKAP79 (Figure 4D). This residue is conserved among MAGUK family members and corresponds to a point mutation in the *Drosophila* protein Discs large that produces a strong mutant phenotype (Woods et al., 1996). This mutation also has been shown to disrupt an intramolecular interaction between the SH3 and GK domains of PSD-95, which does not depend on polyproline sequences (McGee and Bredt, 1999). Thus, AKAP79/150 appears to interact with the SH3 domain of PSD-95 in a manner that is independent of proline-rich sequences.

Association of AKAP79/150 with PSD-95-NR2B Complex In Vivo

PSD-95 is thought to cross-link ion channels like the NMDA receptor to cytoskeletal and signaling elements underlying the postsynaptic density (Sheng and Pak, 1999). The modular nature of the protein ideally suits it for this role. Numerous studies have implicated the N-terminal PDZ domains as sites of interaction with the cytoplasmic tails of ion channels, whereas the C-terminal modules have been shown to mediate association with cytoskeletal and other PSD proteins (reviewed by Fanning and Anderson, 1999). Based on our evidence that AKAP79/150 binds to the SH3 and GK domains of PSD-95, it is reasonable to assume that the PDZ domains are unoccupied and thus are available for interaction with ligands such as NR2 subunits of the NMDA receptor (Kornau et al., 1995). To test this hypothesis, we transfected NR2B, PSD-95, and AKAP79 into COS7 cells and performed coimmunoprecipitation experiments (Figure 5A).

We initially confirmed that the PSD-95-AKAP79 complex could be reconstituted in COS cells. Indeed, antibodies to PSD-95 coimmunoprecipitated AKAP79, whereas nonimmune IgG did not (Figure 5B, left). In reciprocal experiments, antibodies to AKAP79 coimmunoprecipitated PSD-95 (Figure 5B, right). Other control experiments confirmed that NR2B could be coimmunoprecipitated with PSD-95 (Figure 5C), as previously demonstrated (Kim et al., 1996). To test for ternary complex formation between NR2B, PSD-95, and AKAP79, we immunoprecipitated the anchoring protein and looked for both binding partners in the resulting precipitates. PSD-95 (Figure 5D, left) and, more importantly, NR2B (Figure 5D, right) coprecipitated with AKAP79, indicating that a ternary complex forms inside cells. To confirm that these interactions were physiologically relevant, we looked for an association between AKAP150 and NMDA receptors in brain. In coimmunoprecipitation experiments from deoxycholate solubilized synaptic plasma membranes, AKAP150 antibodies, but not control IgG, immunoprecipitated NR2B from extracts (Figure 5E). In addition, we also found NR1 and NR2A subunits in AKAP150 immunoprecipitates (data not shown). In light of our data that PSD-95 associates with AKAP150 in brain (Figure 2D), these results suggest that a ternary complex containing the NMDA receptor, PSD-95, and AKAP150 exists in vivo. Given that AKAP79/150 also associates with the kinases PKA and PKC, this provides a mechanism by which a physiologically relevant substrate, the NMDA receptor, is linked to regulatory enzymes via a protein bridge of PSD-95 and AKAP79/150.

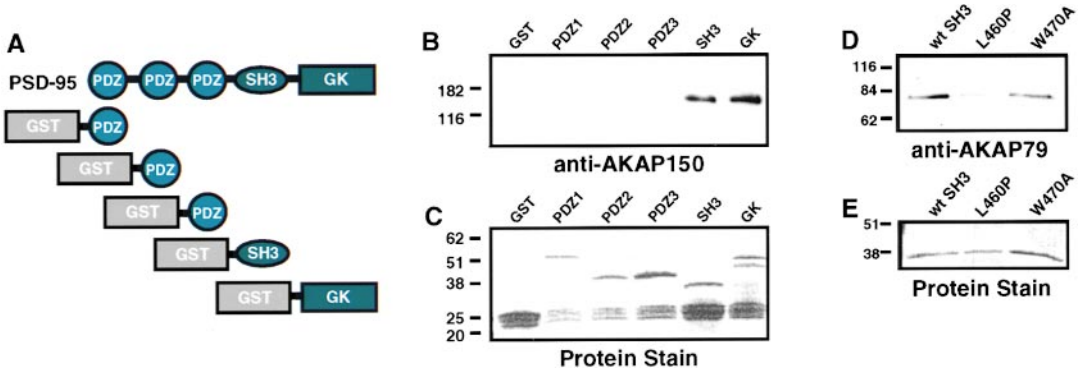

Figure 4. Binding of AKAP150 to SH3 and GK Regions of PSD-95

(A) GST fusion proteins of the individual domains of PSD-95 were constructed for use in pulldown assays. Modular domains are indicated.

(B) Whole rat brain extract was incubated with GST, GST-PDZ1, GST-PDZ2, GST-PDZ3, GST-SH3, or GST-GK beads. Bound proteins were analyzed for the presence of AKAP150 by immunoblotting. AKAP150 was specifically retained by both the SH3 and GK regions but not by control GST or the PDZ domains.

(C) Protein stain of membrane prior to immunoblotting shown in (B) shows that approximately equivalent amounts of each fusion protein were used in pulldown assays.

(D) Specificity of AKAP79 binding to the SH3 domain binding was tested in pulldown experiments using SH3 domain mutants of PSD-95. Binding to L460P mutant was greatly reduced compared to wild-type (wt) SH3. W470A mutation did not alter binding to AKAP79.

(E) Protein stain of membrane prior to immunoblotting shown in (D) shows that equivalent amounts of wild-type and mutant SH3 domain fusion proteins were used in pulldown assays.

AKAP150 Associates with GluR1 in Brain

Recently, a PDZ-mediated interaction between SAP97 and the GluR1 subunit of the AMPA receptor was reported (Leonard et al., 1998). Given that AKAP150 appears to associate with multiple members of the MAGUK family, including SAP97 (Figures 2B and 3B), we were interested in whether it may be involved in directing signaling enzymes to AMPA-type glutamate receptors. We investigated the distribution of GluR1 staining relative to MAGUK protein staining in hippocampal neurons (Figure 6A). GluR1 showed a punctate pattern of labeling that codistributed with MAGUK labeling, suggesting that the proteins are localized together at synaptic sites. Since SAP97 and AMPA receptors are readily extractable in mild detergents (Wenthold et al., 1992; Leonard et al., 1998), we performed coimmunoprecipitation experiments from rat brain solubilized in 1% Triton X-100. In accordance with previous reports (Leonard et al., 1998), a protein corresponding in size to SAP97 (~116 kDa) coimmunoprecipitated with GluR1 (Figure 6B). We did not detect a band corresponding in size to PSD-95 in GluR1 immunoprecipitates using a pan-MAGUK antibody, further supporting the conclusion that the 116 kDa band is SAP97. Importantly, AKAP150 also was specifically detected in GluR1 immunoprecipitates (Figure 6C). In fact, AKAP150 was the only anchoring protein detected in GluR1 immunoprecipitates by RII overlay (data not shown), suggesting that it is likely the predominant AKAP associated with AMPA receptor complexes in brain. In reciprocal experiments, GluR1 was specifically detected in AKAP150 immunoprecipitates (Figure 6D). Importantly, GluR1 was not detected in control immunoprecipitation experiments using nonimmune IgG or antibodies against another synaptic AKAP, yotiao (Figure 6D). We estimate that ~2%–3% of the total GluR1 is found associated with AKAP150 in these experiments. This stoichiometry is reasonable given that isolation of GluR1 with AKAP150 represents a ternary complex, and it is likely that some protein–protein interactions would be disrupted during the homogenization and purification procedures. In addition, AKAP150 functions as a scaffold for signaling enzymes, that, by necessity, act at catalytic levels. Thus, one AKAP150 signaling complex may be positioned in proximity to target enzymes to several AMPA receptors. Taken together, our data suggest that a ternary complex containing GluR1, SAP97, and AKAP150 exists in brain.

AKAP79-Associated GluR1 is Phosphorylated on Ser-845

The GluR1 subunit of AMPA receptors is phosphorylated by PKA on serine 845 (Ser-845; Roche et al., 1996; Mammen et al., 1997). PKA phosphorylation potentiates AMPA receptor activity in recombinant systems (Keller et al., 1992; Roche et al., 1996) and in cultured hippocampal neurons (Greengard et al., 1991; Wang et al., 1991). In addition, AKAPs have been implicated in the PKA-mediated modulation of native AMPA receptor function (Rosenmund et al., 1994). We were therefore interested in whether AKAP79-dependent targeting of PKA to GluR1 might function to regulate the phosphorylation state of Ser-845. As a prelude to these studies, we reconstituted the GluR1-SAP97-AKAP79 complexes in COS7 cells. As expected, SAP97 was detected in AKAP79 immunoprecipitates (Figure 7A, left), and AKAP79 coprecipitated with SAP97 in reciprocal experiments (Figure 7A, right). When all three cDNAs were transfected into COS7 cells, antibodies against Myc-tagged SAP97 were able to coimmunoprecipitate GluR1 (Figure 7B, left). More importantly, AKAP79 antibodies also coimmunoprecipitated GluR1, indicating that a ternary complex containing GluR1, SAP97, and AKAP79 forms inside cells (Figure 7B, right).

Having established that we were able to assemble a SAP97-AKAP79 complex on the ion channel, we were in a position to test the functional consequence of targeting PKA to GluR1. Toward this end, we used

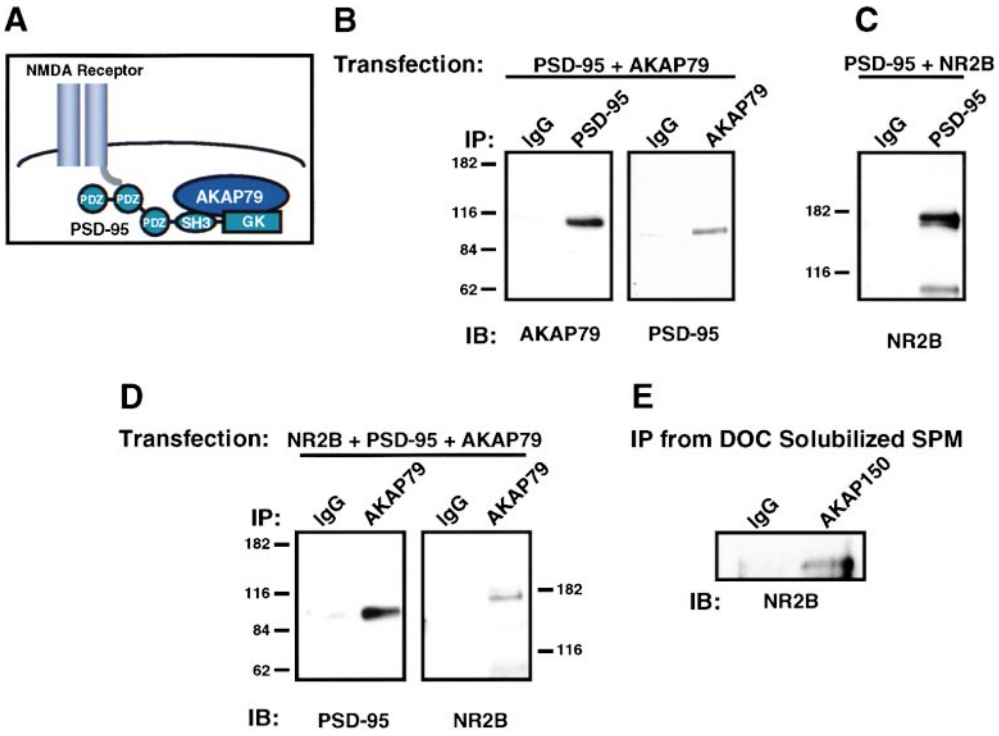

Figure 5. Formation of NR2B-PSD-95-AKAP79 Complex inside Cells and Association of NR2B with AKAP150 in Brain

(A) Schematic diagram of proposed NMDA receptor-PSD-95-AKAP complex.

(B) Association of PSD-95 and AKAP79 in transfected cells. Extracts from COS7 cells transfected with Myc-PSD-95 and GFP-AKAP79 were immunoprecipitated with PSD-95, AKAP79, or control IgG antibodies. PSD-95 immunoprecipitates were blotted with AKAP79 antibodies (left), and AKAP79 immunoprecipitates were blotted with PSD-95 antibodies (right). PSD-95 coimmunoprecipitated AKAP79 and vice versa.

(C) Association of PSD-95 and NR2B in transfected cells. Extracts from COS7 cells transfected with Myc-PSD-95 and NR2B were immunoprecipitated with PSD-95 or control IgG antibodies. Immunoprecipitates were blotted with NR2B antibodies. PSD-95 coimmunoprecipitated NR2B.

(D) Association of NR2B-PSD-95-AKAP79 complex in transfected cells. Extracts from COS7 cells transfected with NR2B, Myc-PSD-95 and GFP-AKAP79 were immunoprecipitated with AKAP79 or control IgG antibodies. AKAP79 immunoprecipitates were blotted with PSD-95 (left) and NR2B antibodies (right). AKAP79 coimmunoprecipitated both PSD-95 and NR2B.

(E) Association of NMDA receptor and AKAP150 in brain. Extracts of rat forebrain synaptosomal membranes were immunoprecipitated with AKAP150 or control nonimmune IgG antibodies. NR2B coimmunoprecipitated with AKAP150.

phosphospecific antibodies generated against Ser-845 (Mammen et al., 1997) to monitor the phosphorylation state of the channel (Figures 7C and 7D). Cells cotransfected with GluR1, SAP97, and AKAP79 were treated with forskolin and IBMX to stimulate activation of PKA by cAMP. Extracts were immunoprecipitated with AKAP79 antibodies to isolate the population of GluR1 associated with the AKAP. Approximately equivalent amounts of GluR1 were precipitated by AKAP79 antibodies in all conditions (Figure 7C, bottom). Upon PKA stimulation, phosphorylation of Ser-845 was significantly increased ($n = 3$, $p < 0.05$) in cells containing wild-type AKAP79 (Figures 7C [top] and 7D). As a control for these experiments, we used a mutant form of AKAP79 in which a proline was inserted into the PKA binding site (AKAP79-Pro) to prevent PKA binding (Gao et al., 1997). Importantly, in cells transfected with the AKAP79-Pro mutant, enhancement of Ser-845 phosphorylation was significantly reduced in response to PKA stimulation ($n = 4$, $p < 0.01$; Figures 7C [top] and 7D). Together, these data indicate that targeting of PKA to GluR1 through association with AKAP79 facilitates phosphorylation of Ser-845.

To test whether SAP97 is required for this AKAP79-mediated phosphorylation of Ser-845, we repeated these experiments using a point mutant of GluR1 that should disrupt interaction with the PDZ domains of SAP97. The PDZ consensus motif at the extreme C terminus of the ion channel was changed from TGL to AGL (T887A). In control experiments, this mutant form of GluR1 was no longer precipitated with SAP97, indicating that threonine 887 indeed is required for interaction with SAP97 (Figure 7E, left). Importantly, the T887A mutation does not affect the ability of a C-terminal antibody to recognize GluR1 (Figure 7E, extract ["Ex"] lanes). In addition, unlike wild-type GluR1 (Figure 7B, right), GluR1-AGL did not coimmunoprecipitate with AKAP79 (Figure 7E, right). These data indicate that a PDZ-mediated interaction between SAP97 and GluR1 is required for association with the AKAP, providing further evidence for the specificity of the interaction.

To test whether disruption of the GluR1-SAP97 interaction affects Ser-845 phosphorylation, we transfected wild-type (TGL) or mutant (AGL) GluR1, SAP97, and AKAP79 into COS cells, immunoprecipitated GluR1, and examined Ser-845 phosphorylation under basal conditions. Phosphorylation of Ser-845 was significantly reduced ($n = 4$, $p < 0.05$) in cells transfected with the AGL mutant compared to cells transfected with the wild-type receptor (Figures 7F and 7G). These results suggest

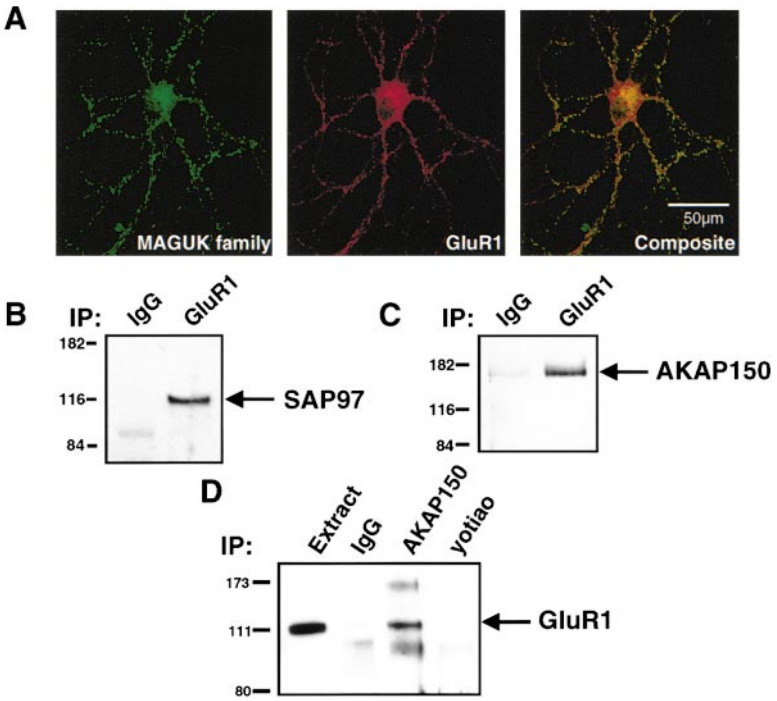

Figure 6. AKAP150 Associates with GluR1 in Brain

(A) Distribution of GluR1 and MAGUK proteins in hippocampal neurons. Three-week-old cultured hippocampal neurons were labeled simultaneously with antibodies against MAGUK family proteins (left) and GluR1 (middle). GluR1 exhibits a punctate pattern of distribution along dendrites that codistributes with MAGUK labeling (right).

(B) Coimmunoprecipitation of SAP97 with GluR1 from brain. Extracts from whole rat brain were immunoprecipitated with GluR1 or control IgG antibodies and blotted for SAP97 with pan-MAGUK antibodies. SAP97 was coimmunoprecipitated with GluR1 antibodies but not with control IgG.

(C) Coimmunoprecipitation of AKAP150 with GluR1 from brain. Extracts from whole rat brain were immunoprecipitated with GluR1 or control IgG antibodies and blotted with AKAP150 antibodies. AKAP150 was present in GluR1, but not control IgG, precipitates.

(D) Coimmunoprecipitation of GluR1 with AKAP150 from brain. Brain extracts were immunoprecipitated with control IgG, AKAP150, or yotiao antibodies. GluR1 was present only in AKAP150 precipitates.

that a PDZ-mediated interaction enhances basal phosphorylation of Ser-845, a known PKA site in GluR1. Together, these cell-based studies suggest that targeting of PKA to GluR1 via a SAP97-AKAP79 protein bridge facilitates phosphorylation of Ser-845.

Discussion

Phosphorylation of glutamate receptors is a critical regulatory event in the control of synaptic function and plasticity (reviewed by Dingledine et al., 1999). In this report, we provide evidence for the existence of a macromolecular transduction unit in which PKA is targeted to glutamate receptors through the direct interaction of two distinct sets of synaptic organizing molecules—the MAGUK proteins and AKAP79/150. These interactions increase the complexity of signaling networks at excitatory synapses and may provide a structural framework that permits preferential targeting of kinases to glutamate receptors. Presumably, such a highly organized kinase–substrate complex ensures rapid and efficient phosphorylation of ion channels in response to local synaptic signals.

The MAGUK proteins provide the central scaffold upon which the complex is assembled. The N-terminal PDZ domains of PSD-95 and SAP97, two members of the MAGUK protein family, bind to the tails of NMDA and AMPA receptor subunits, respectively (Kornau et al., 1995; Leonard et al., 1998). Our data suggest that AKAP79/150 and its associated kinases can be recruited to these glutamate receptor complexes via interaction with the C-terminal SH3 and GK domains of the MAGUKs. The demonstration that two independent sites of contact mediate interaction between AKAP79/150 and MAGUK proteins is interesting in light of other mapping studies that have defined linear sequences of 4–6 amino acids as ligands for PDZ, SH2, and SH3 domains (Pawson and Scott, 1997). Certainly, multiple sites of contact are not unprecedented and are likely to provide additional stability to a given protein complex. For example, the KA2 subunit of the kainate receptor, like AKAP79, binds to both the SH3 and GK domains of PSD-95. In addition, AKAP79/150 appears to bind to the β2 adrenergic receptor through sites in both the third intracellular loop and the C-terminal tail (Fraser et al., 2000). Interestingly, mutations in the SH3 and GK domains of the *Drosophila* MAGUK Discs large produce severe phenotypes, suggesting that these modules mediate interactions that are critical for regulating MAGUK function (Woods et al., 1996). Furthermore, deletion of these regions of PSD-95 in mice produces defects in synaptic plasticity that have been attributed to altered downstream signaling events (Migaud et al., 1998). A potential explanation for these observations is that the AKAP79/150 signaling scaffold no longer can be recruited to glutamate receptors through interaction with MAGUK proteins.

AKAP79/150 previously has been shown to provide a scaffold for three signaling enzymes—PKA, PKC, and calcineurin (Klauck et al., 1996). Interestingly, we show here that PSD-95 competes with calcineurin for binding to AKAP79/150 in vitro. Preliminary mapping experiments suggest that the two proteins do not share the same binding site on the AKAP, as a deletion mutant that does not bind to calcineurin still binds to PSD-95 (M. L. Dell'Acqua, M. C., and J. D. S., unpublished data). Thus, a more likely explanation is that PSD-95 binding to AKAP79/150 sterically hinders interaction with calcineurin. These data support the notion that, when bound to MAGUKs, AKAP79/150 may preferentially target kinases but not phosphatases to certain glutamate receptors at the PSD. This could provide a mechanism to favor ion channel phosphorylation through preferential recruitment of regulatory kinases.

While our results clearly argue for a role for anchored PKA in receptor phosphorylation, targeted phosphatases are also certain to participate in receptor dephosphorylation. However, we have shown that interaction

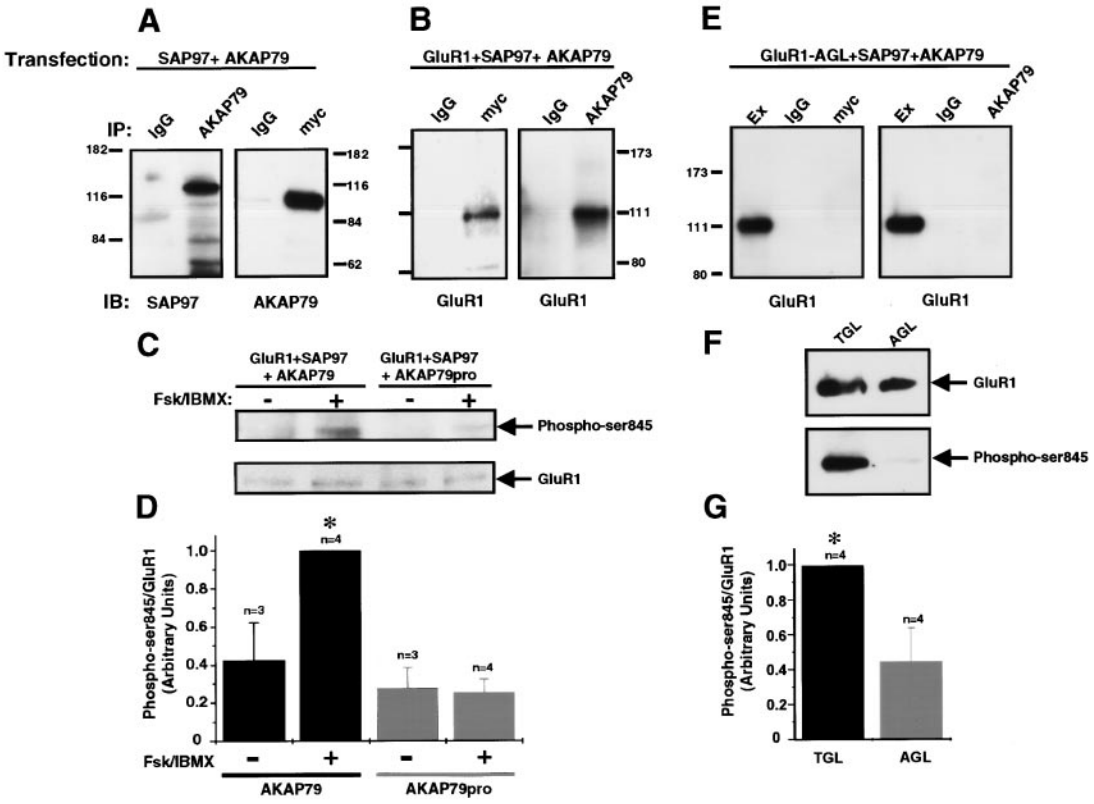

Figure 7. Phosphorylation of Ser-845 of GluR1 by AKAP79-Anchored PKA and PDZ Domain Interaction with SAP97

(A) Association of SAP97 and AKAP79 in transfected cells. Extracts from COS7 cells transfected with Myc-SAP97 and GFP-AKAP79 were immunoprecipitated with AKAP79, SAP97 (Myc tag), or control IgG antibodies. AKAP79 immunoprecipitates were blotted for SAP97 with pan-MAGUK antibodies (left), and SAP97 immunoprecipitates were blotted with AKAP79 antibodies (right panel). AKAP79 coimmunoprecipitated SAP97 and vice versa.

(B) Association of GluR1-SAP97-AKAP79 complex in transfected cells. Extracts from COS7 cells transfected with GluR1, myc-SAP97, and GFP-AKAP79 were immunoprecipitated with SAP97 (Myc), AKAP79, or control IgG antibodies. Both SAP97 (left) and AKAP79 immunoprecipitates (right) were blotted with GluR1 antibodies. GluR1 was coimmunoprecipitated with both SAP97 and AKAP79.

(C) Phosphorylation of AKAP79-associated GluR1 by PKA. COS7 cells transfected with GluR1, Myc-SAP97, and either wild-type GFP-AKAP79 or GFP-AKAP79-Pro (a mutant that does not bind to PKA) were treated with (+) or without (−) 20 μM forskolin (Fsk) and 75 μM isobutylmethylxanthine (IBMX) to stimulate PKA. Extracts were immunoprecipitated with antibodies against AKAP79. Phosphorylation of Ser-845 was analyzed using phosphorylation site-specific antibody (top). The total amount of GluR1 was analyzed using an antibody against the C terminus of GluR1 (bottom). Stimulation of PKA increases the phosphorylation of GluR1 on Ser-845 in complexes containing wild-type AKAP79. In contrast, when associated with AKAP79-Pro, which does not bind to PKA, cAMP-mediated phosphorylation of Ser-845 was diminished.

(D) PKA anchoring–defective mutant of AKAP79 signficantly reduces cAMP enhancement of GluR1 on Ser-845. Results from several experiments (number indicated above each bar) were combined. The relative amount of GluR1 phosphorylation on Ser-845 was analyzed by determining the ratio of signals for the phospho-Ser-845 antibody and the C-terminal antibody for each experimental condition. Stimulation of PKA with Fsk/IBMX signficantly increased Ser-845 phosphorylation in complexes containing wild-type AKAP79 ($n = 3$, $p < 0.05$). Substitutution of AKAP79-Pro in the complex reduced this cAMP-mediated enhancement of Ser-845 phosphorylation ($n = 4$, $p < 0.01$).

(E) Formation of GluR1-SAP97-AKAP79 complex requires C-terminal PDZ binding motif. COS7 cells were transfected with a C-terminal AGL mutant of GluR1, Myc-SAP97, and GFP-AKAP79. Extracts were immunoprecipitated with control IgG, Myc, or AKAP79 antibodies and blotted for GluR1. GluR1-AGL was not immunoprecipitated either with SAP97 (left) or with AKAP79 (right). The AGL mutant is still recognized by the C-terminal GluR1 antibody (extract [Ex] lanes).

(F) Disruption of PDZ binding site reduces basal phosphorylation of GluR1 Ser-845. COS7 cells were transfected with either wild-type (TGL) or mutant (AGL) GluR1, Myc-SAP97, and GFP-AKAP79, and extracts were immunoprecipitated with GluR1 antibodies. The total amount of GluR1 was analyzed using an antibody against the C terminus of GluR1 (top), and the basal level of phosphorylation of Ser-845 was analyzed using a phosphorylation site-specific antibody (bottom). Phosphorylation of Ser-845 was greatly diminished on GluR1-AGL compared to wild-type GluR1-TGL.

(G) PDZ interaction point mutation in GluR1 significantly reduces Ser-845 phosphorylation. Results from several ($n = 4$) experiments were combined. The relative amount of GluR1 phosphorylation on Ser-845 was analyzed by determining the ratio of signals for the phospho-Ser-845 antibody and the C-terminal antibody for each experimental condition. Insertion of a point mutant (T887A) in the PDZ ligand site of GluR1, which disrupts interaction with SAP97-AKAP79 complex, significantly reduces phosphorylation of Ser-845 ($p < 0.05$).

of AKAP79 with MAGUKs appears to exclude the phosphatase calcineurin from the complex. One possibility is that phosphatases may be recruited to AMPA receptors through anchoring proteins other than AKAP79/150. In fact, recent reports suggest that the phosphatase PP1 may be targeted to AMPA receptor complexes through its association with spinophilin (Yan et al., 1999). Kinase–phosphatase targeting to some NMDA receptors may

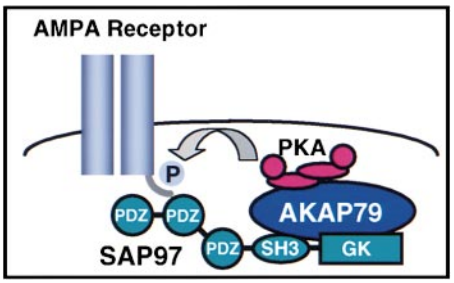

Figure 8. Protein–Protein Interactions Direct PKA to AMPA Receptors

SAP97 binds to the C terminus of GluR1 via a PDZ domain-TGL interaction. Through its SH3 and GK domains, SAP97 also interacts with AKAP79. Formation of a ternary complex containing GluR1, SAP97, and AKAP79 positions PKA near the receptor where it can phosphorylate serine 845, a known PKA site in GluR1.

be more direct. Through interaction with the NR1-1A splice variant, the anchoring protein yotiao targets both PKA and active PP1 to NMDA receptor complexes, conferring bidirectional regulation of NMDA receptor activity (Westphal et al., 1999). When considered in light of our present data, this raises the intriguing possibility that signaling enzymes may be recruited to certain NMDA receptors through simultaneous association with two anchoring proteins: yotiao and AKAP79.

Phosphorylation of the cytoplasmic tail of GluR1 potentiates receptor function (Keller et al., 1992; Roche et al., 1996). CaMKII increases the unitary channel conductance via phosphorylation of Ser-831 (Barria et al., 1997; Derkach et al., 1999), while PKA phosphorylation of Ser-845 increases the peak open probability (Banke et al., 2000). Phosphorylation-dependent changes in AMPA receptor activity have been proposed to underlie some aspects of LTP and LTD (reviewed by Malenka and Nicoll, 1999). For example, CamKII phosphorylation appears to be essential for the induction of hippocampal LTP (Lisman, 1994; Malenka and Nicoll, 1999; Soderling and Derkach, 2000), while recent studies have implicated a role for PKA in LTD (Kameyama et al., 1998; Lee et al., 1998). Our present results suggest that AKAP79/150 functions as an important player in the postsynaptic regulation of excitatory transmission by targeting PKA to AMPA receptors. Specifically, we have shown that cAMP-dependent phosphorylation of Ser-845, a known PKA site in GluR1, is enhanced when the kinase is targeted to the channel via a SAP97-AKAP79 complex. This enhancement in phosphorylation is significantly reduced when a PKA anchoring–defective form of AKAP79 is substituted in the complex. Furthermore, a mutation in the PDZ binding site in the tail of GluR1, which uncouples the receptor from SAP97, reduces the basal level of phosphorylation of Ser-845 compared to wild-type GluR1. Together, our results suggest that phosphorylation of Ser-845 is mediated through a SAP97-AKAP79 complex that targets PKA to GluR1 via a PDZ domain interaction (Figure 8). This is particularly interesting in light of recent evidence implicating a GluR1-PDZ domain interaction in the delivery of AMPA receptors into synapses (Hayashi et al., 2000). Our data suggest that recruitment of a SAP97-AKAP79-PKA complex may play a role in this process. Manipulation of these protein–protein interactions in animals should provide models to study the role of this synaptic signaling unit in regulating glutamate receptor function in vivo.

Experimental Procedures

Antibodies

The following primary antibodies were used for immunoblotting: rabbit polyclonal to AKAP150 (clone 4361J from ICOS, 9.8 μg/ml, 1:3000 dilution), rabbit polyclonal to AKAP79 (918I clone from ICOS, 5.6 μg/ml, 1:2500), mouse monoclonal to PKA catalytic subunit (clone 5B, Transduction Laboratories, 1:1000 dilution), rabbit polyclonal against PKA type II regulatory subunit (clone 6825, 1:1000 dilution), mouse monoclonal to PKCα (Transduction Laboratories, clone 3, 1:1000 dilution), mouse monoclonal to PSD-95 (clone K28/43 from Upstate Biotechnology, 1:10,000 dilution), mouse monoclonal to PSD-95 family (MAGUK family, Upstate Biotechnology, 1:2500 dilution), rabbit polyclonal to GluR1 (Upstate Biotechnology, 1:1000 dilution), rabbit polyclonal to NR2B (Upstate Biotechnology, 1:500 dilution), mouse monoclonal to the Myc tag (Santa Cruz, 1:500), mouse monoclonal to synaptophysin (Sigma S-5768, 1:1000), rabbit polyclonal to WAVE (VO59, 1:500), and rabbit polyclonal phosphospecific antibody against Ser-845 of GluR1 (Mammen et al., 1997; 1:100–1:250).

Expression Constructs

For construction of GST fusion proteins of PSD-95, the individual domains (PDZ1, amino acids [aa] 2–155; PDZ2, aa 156–276; PDZ3, aa 277–415; SH3, aa 416–511; GK, aa 512–744) were PCR amplified using rat PSD-95 in pGEX-2T as a template and subcloned into BamH1–EcoR1 (PDZ1, PDZ2, PDZ3, and SH3) or BglII–EcoRI (GK) sites in pGEX-2TK. The L460P mutation in the SH3 domain of PSD-95 was made using the QuikChange mutagenesis method (Stratagene). Construction of His-tagged AKAP79 in the pET16 vector and AKAP79:GFP and AKAP79-Pro:GFP in the pEGFPN1 vector (Clontech) has been described (Gao et al., 1997; Dell'Acqua et al., 1998). The T887A mutation at the C terminus of GluR1 was made by site-directed mutagenesis using the TOPO TA cloning kit (Invitrogen). GST-PSD-95 in pGEX-2T, GST-SAP97 in pGEX-2TN, GST-SH3 (W470A) in pGEX4T-1, and the Myc-tagged SAP97 construct were generously provided by Craig Garner. The Myc-tagged PSD-95 construct was a gift from Morgan Sheng, and the NR2B expression construct in pCDNA3 was a gift from Gary Westbrook. The GluR1 mammalian expression construct in pRK5 has been described (Sommer et al., 1990).

RII Overlay Assay

PSD fractions were prepared from frozen rat brains as previously described (Cho et al., 1992). PKA binding proteins were detected by the RII overlay procedure (Hausken et al., 1998). Briefly, PSD fractions were separated by SDS–PAGE on a 4%–15% gradient gel, transferred to nitrocellulose membranes, and incubated overnight in 5 ml 0.1% Blotto (TBST, 5% milk, and 0.1% BSA) containing 5 μl [^{32}P]RII (~200,000 cpm/μl). The membrane was washed extensively with TBST and exposed to autoradiographic film.

Hippocampal Neuron Culture, Immunocytochemistry, and Confocal Microscopy

Cultured rat neonatal hippocampal neurons were grown on coverslips suspended above a glial cell feeder layer. Three weeks following plating, neurons were processed for antibody labeling. Neurons were fixed in 3.7% formaldehyde, permabilized in 0.2% Triton X-100, blocked in 0.2% BSA in PBS, and incubated with primary antibodies (mouse monoclonal to PSD-95, clone 6G6-1C9 from Affinity Bioreagents, 1:200; rabbit polyclonal antibody to AKAP150, clone 4361J, 1:500; rabbit polyclonal to GluR1 from Upstate Biotechnology, 1:100; or mouse monoclonal to PSD-95 family from Upstate Biotechnology, 1:100). FITC-conjugated anti-mouse (1:200), Cy5-conjugated anti-rabbit (1:500), or Texas Red–conjugated anti-rabbit (1:200) secondary antibodies from Jackson Immunoresearch were used. Antibody labeling was analyzed with a Bio-Rad confocal microscope (Fraser et al., 1998).

Brain Extract Preparations and Immunoprecipitations

For immunoprecipitation of PSD-95-AKAP150 and AKAP150-NR2B complexes, extracts of rat forebrain crude synaptosomes were prepared by solubilization in deoxycholic acid according to the procedure of Dunah et al. (1998). Forebrain P2 fraction was extracted in 1% deoxycholic acid and dialyzed overnight against 0.1% Triton X-100 and 50 mM Tris (pH 7.4). Following centrifugation at 38,000 × g for 30 min at 4°C, 100 μg of supernatant was incubated with 4 μg of control nonimmune IgG, PSD-95 or AKAP150 antibodies and 40 μl of protein A-sepharose overnight at 4°C. Beads were washed four times with 1 ml of dialysis buffer. Bound proteins were eluted with SDS-sample buffer, separated by SDS-PAGE on a 4%–15% gradient gel (Biorad), transferred to nitrocellulose membrane and detected by immunoblotting using ECL reagents (Pierce).

For immunoprecipitations of GluR1/SAP97/AKAP150 complexes, one frozen rat brain (Pelfreeze) was homogenized in 5 ml HSE buffer (20 mM HEPES [pH 7.4], 150 mM NaCl, 5 mM EDTA, containing protease inhibitors as above) and centrifuged at 38,000 × g for 30 min. The pellet was resuspended in 5 ml HSE buffer containing 1% Triton X-100 and centrifuged for an additional 30 min at 38,000 × g. Soluble extracts (500 μl) from the second spin were precipitated by adding 4 μg of control nonimmune IgG, GluR1, AKAP150 or yotaio (VO69 [Westphal et al., 1999]) antibodies and 40 μl of protein A-sepharose that had been prewashed three times in IP buffer (10 mM phosphate, 150 mM NaCl, 5 mM EDTA, 5 mM EGTA with 1% Triton X-100). Following overnight incubation at 4°C, beads were washed twice in IP buffer with 1% Triton X-100, twice in the same buffer with 650 mM NaCl, and twice in IP buffer without detergent. Bound proteins were detected by immunoblotting.

For immunoprecipitation from heterologous cells, COS7 cells were transfected at 50%–70% confluency in 10 cm plates using the LipofectAMINE PLUS kit (Gibco-BRL), according to manufacturer's instructions, using 4 μg of each cDNA construct per plate. Cells were harvested and lysed 24 hr after transfection in 400 μl IP buffer containing 1% Triton X-100 and protease inhibitors. Supernatants were incubated with 4 μg antibody or control nonimmune IgG and 40 μl of prewashed protein A- or G-agarose beads. Following overnight incubation at 4°C, the immunoprecipitates were washed as descibed above with IP buffers. Bound proteins were analyzed by immunoblotting.

cAMP Agarose Purification

For cAMP agarose experiments, rat brain extracts were prepared by Dounce homogenizing frozen rat brains (Pelfreeze) in lysis buffer (20 mM HEPES [pH 7.4], 20 mM NaCl, 5 mM EDTA, 5 mM EGTA, 1.0% Triton X-100, 1 mM benzamidine, 2 μg/ml leupeptin, 2 μg/ml pepstatin, and 100 mM AEBSF) and centrifuging at 38,000 × g for 30 min. Extracts were incubated with cAMP agarose for 18 hr at 4°C in the presence or absence of 50 mM cAMP (Sigma). The resin was washed twice with hypotonic buffer (10 mM HEPES [pH 7.4], 1.5 mM $MgCl_2$, 10 mM KCl, 0.1% NP-40, 1 mM DTT, 1 mM benzamidine, 2 μg/ml leupeptin, 2 μg/ml pepstatin, and 100 mM AEBSF) containing 500 mM NaCl and twice with hypotonic buffer without NaCl. Proteins were eluted from the resin by incubation with 75 mM cAMP for 1 hr at room temperature. Eluted proteins were precipitated with TCA, resuspended in SDS sample buffer, and analyzed by immunoblotting.

GST Pulldowns

For GST pulldown experiments, rat brain extract was prepared as described above for the cAMP agarose experiments. Beads containing GST fusion proteins were incubated with 500 μl of extract and incubated overnight at 4°C. The beads were washed three times in 10 mM phosphate, 1 M NaCl with 1% Triton X-100, twice with the same buffer containing 150 mM NaCl, and once with PBS. Bound proteins were analyzed by immunoblotting.

Calcineurin Competition Binding Assay

Beads charged with GST-PSD-95 (or control GST) were incubated with 0.2 μg (11 nM) purified recombinant AKAP79 in the presence of increasing concentrations of purified calcineurin A (a generous gift of Tom Soderling)—0, 100, 200, or 400 nM or 400 nM control BSA in 400 μl IP buffer overnight at 4°C. The presence of AKAP79 bound to the beads was analyzed by immunoblotting.

GluR1 Phosphorylation and Immunoprecipitation from COS7 Cells

For GluR1 phosphorylation experiments, 24 hr following transfection, cells were treated with 20 μM forskolin FSK and 75 μM IBMX for 15 min. Cells were rinsed in PBS and scraped into 1 ml of IP buffer containing protease inhibitors plus phosphatase inhibitors (10 mM sodium pyrophosphate, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 1 μM okadaic acid, 1μM microcystin, and 1 μM cyclosporin). Cells were sonicated and centrifuged at 38,000 × g for 30 min. The membrane pellets were resuspended in 200 μl IP buffer containing 1% SDS and then diluted with 800 μl IP buffer containing 1% Triton X-100. Extracts were then centrifuged at 14,000 × g for 15 min, and the supernatants were subjected to immunoprecipitation with AKAP79 antibodies as described above and analyzed by Western blotting using GluR1 phosphospecific antibody to Ser-845. Phospho-Ser-845 and GluR1 signals were quantitated using NIH Image and the ratio was determined. The results of several experiments were combined and subjected to statistical analyses.

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References

Anderson, J.M. (1996). Cell signalling: MAGUK magic. Curr. Biol. *6,* 382–384.

Banke, T.G., Bowie, D., Lee, H., Huganir, R.L., Schousboe, A., and Traynelis, S.F. (2000). Control of GluR1 AMPA receptor function by cAMP-dependent protein kinase. J. Neurosci. *20,* 89–102.

Barria, A., Derkach, V., and Soderling, T. (1997). Identification of the Ca^{2+}/calmodulin-dependent protein kinase II regulatory phosphorylation site in the alpha-amino-3-hydroxyl-5-methyl- 4-isoxazole-propionate-type glutamate receptor. J. Biol. Chem. *272,* 32727–32730.

Carr, D.W., Stofko-Hahn, R.E., Fraser, I.D.C., Cone, R.D., and Scott, J.D. (1992). Localization of the cAMP-dependent protein kinase to the postsynaptic densities by A-kinase anchoring proteins: characterization of AKAP79. J. Biol. Chem. *24,* 16816–16823.

Cho, K.O., Hunt, C.A., and Kennedy, M.B. (1992). The rat brain postsynaptic density fraction contains a homolog of the Drosophila discs-large tumor suppressor protein. Neuron *9,* 929–942.

Coghlan, V.M., Perrino, B.A., Howard, M., Langeberg, L.K., Hicks, J.B., Gallatin, W.M., and Scott, J.D. (1995). Association of protein kinase A and protein phosphatase 2B with a common anchoring protein. Science *267,* 108–112.

Colledge, M., and Scott, J.D. (1999). AKAPs: from structure to function. Trends Cell Biol. *9,* 216–221.

Dell'Acqua, M.L., Faux, M.C., Thorburn, J., Thorburn, A., and Scott, J.D. (1998). Membrane targeting sequences on AKAP79 bind phosphatidylinositol-4,5-bisphosphate. EMBO J. *17*, 2246–2260.

Derkach, V., Barria, A., and Soderling, T.R. (1999). Ca^{2+}/calmodulin-kinase II enhances channel conductance of alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate type glutamate receptors. Proc. Natl. Acad. Sci. USA *96,* 3269–3274.

Dingledine, R., Borges, K., Bowie, D., and Traynelis, S.F. (1999). The glutamate receptor ion channels. Pharmacol. Rev. *51,* 7–61.

Dong, H., O'Brien, R.J., Fung, E.T., Lanahan, A.A., Worley, P.F., and Huganir, R.L. (1997). GRIP: a synaptic PDZ domain-containing protein that interacts with AMPA receptors. Nature *386,* 279–284.

Dong, H., Zhang, P., Liao, D., and Huganir, R.L. (1999). Characterization, expression, and distribution of GRIP protein. Ann. N Y Acad. Sci. *868,* 535–540.

Dunah, A.W., Luo, J., Wang, Y.H., Yasuda, R.P., and Wolfe, B.B. (1998). Subunit composition of N-methyl-D-aspartate receptors in the central nervous system that contain the NR2D subunit. Mol. Pharmacol. *53,* 429–437.

Erpel, T., Superti-Furga, G., and Courtneidge, S.A. (1995). Mutational analysis of the Src SH3 domain: the same residues of the ligand binding surface are important for intra- and intermolecular interactions. EMBO J. *14,* 963–975.

Fanning, A.S., and Anderson, J.M. (1999). Protein modules as organizers of membrane structure. Curr. Opin. Cell Biol. *11,* 432–439.

Faux, M.C., Rollins, E.N., Edwards, A.S., Langeberg, L.K., Newton, A.C., and Scott, J.D. (1999). Mechanism of A-kinase-anchoring protein 79 (AKAP79) and protein kinase C interaction. Biochem. J. *343,* 443–452.

Fraser, I.D.C., Tavalin, S.J., Lester, L.B., Langeberg, L.K., Westphal, A.M., Dean, R.A., Marrion, N.V., and Scott, J.D. (1998). A novel lipid-anchored A-kinase anchoring protein facilitates cAMP-responsive membrane events. EMBO J. *17,* 2261–2272.

Fraser, I., Cong, M., Kim, J., Rollins, E., Daaka, Y., Lefkowitz, R., and Scott, J. (2000). Assembly of an AKAP/β2-adrenergic receptor signaling complex facilitates receptor phosphorylation and signaling. Curr. Biol., in press.

Gao, T., Yatani, A., Dell'Acqua, M.L., Sako, H., Green, S.A., Dascal, N., Scott, J.D., and Hosey, M.M. (1997). cAMP-dependent regulation of cardiac L-type Ca^{2+} channels requires membrane targeting of PKA and phosphorylation of channel subunits. Neuron *19*, 185–196.

Glantz, S.B., Amat, J.A., and Rubin, C.S. (1992). cAMP signaling in neurons: patterns of neuronal expression and intracellular localization for a novel protein, AKAP 150, that anchors the regulatory subunit of cAMP-dependent protein kinase IIb. Mol. Biol. Chem. *3,* 1215–1228.

Greengard, P., Jen, J., Nairn, A.C., and Stevens, C.F. (1991). Enhancement of glutamate response by cAMP-dependent protein kinase in hippocampal neurons. Science *253,* 1135–1138.

Hausken, Z.E., Coghlan, V.M., Hasting, C.A.S., Reimann, E.M., and Scott, J.D. (1994). Type II regulatory subunit (RII) of the cAMP dependent protein kinase interaction with A-kinase anchor proteins requires isoleucines 3 and 5. J. Biol. Chem. *269,* 24245–24251.

Hausken, Z.E., Coghlan, V.M., and Scott, J.D. (1998). Overlay, ligand blotting and band-shift techniques to study kinase anchoring. In Protein Targeting Protocols, R.A. Clegg, ed. (Totowa, NJ: Humana Press), pp. 47–64.

Hayashi, Y., Shi, S.H., Esteban, J.A., Piccini, A., Poncer, J.C., and Malinow, R. (2000). Driving AMPA receptors into synapses by LTP and CaMKII: requirement for GluR1 and PDZ domain interaction. Science *287,* 2262–2267.

Kameyama, K., Lee, H.K., Bear, M.F., and Huganir, R.L. (1998). Involvement of a postsynaptic protein kinase A substrate in the expression of homosynaptic long-term depression. Neuron *21,* 1163–1175.

Kashishian, A., Howard, M., Loh, C., Gallatin, W.M., Hoekstra, M.F., and Lai, Y. (1998). AKAP79 inhibits calcineurin through a site distinct from the immunophilin-binding region. J. Biol. Chem. *273,* 27412–27419.

Keller, B.U., Hollmann, M., Heinemann, S., and Konnerth, A. (1992). Calcium influx through subunits Glur1/GluR3 of kainate/AMPA receptor channels is regulated by cAMP dependent protein kinase. EMBO J. *11,* 891–898.

Kennedy, M.B. (1997). The postsynaptic density at glutamatergic synapses. Trends Neurosci. *20,* 264–268.

Kim, E., Cho, K.O., Rothschild, A., and Sheng, M. (1996). Heteromultimerization and NMDA receptor-clustering activity of Chapsyn-110, a member of the PSD-95 family of proteins. Neuron *17,* 103–113.

Klauck, T.M., Faux, M.C., Labudda, K., Langeberg, L.K., Jaken, S., and Scott, J.D. (1996). Coordination of three signaling enzymes by AKAP79, a mammalian scaffold protein. Science *271,* 1589–1592.

Kornau, H.C., Schenker, L.T., Kennedy, M.B., and Seeburg, P.H. (1995). Domain interaction between NMDA receptor subunits and the postsynaptic density protein PSD-95. Science *269,* 1737–1740.

Lau, L.F., Mammen, A., Ehlers, M.D., Kindler, S., Chung, W.J., Garner, C.C., and Huganir, R.L. (1996). Interaction of the N-methyl-D-aspartate receptor complex with a novel synapse-associated protein, SAP102. J. Biol. Chem. *271,* 21622–21628.

Lee, H.K., Kameyama, K., Huganir, R.L., and Bear, M.F. (1998). NMDA induces long-term synaptic depression and dephosphorylation of the GluR1 subunit of AMPA receptors in hippocampus. Neuron *21,* 1151–1162.

Leonard, A.S., Davare, M.A., Horne, M.C., Garner, C.C., and Hell, J.W. (1998). SAP97 is associated with the alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptor GluR1 subunit. J. Biol. Chem. *273,* 19518–19524.

Lisman, J. (1994). The CaM kinase II hypothesis for the storage of synaptic memory. Trends Neurosci. *17,* 406–412.

Malenka, R.C., and Nicoll, R.A. (1999). Long-term potentiation—a decade of progress? Science *285,* 1870–1874.

Mammen, A.L., Kameyama, K., Roche, K.W., and Huganir, R.L. (1997). Phosphorylation of the alpha-amino-3-hydroxy-5-methylisoxazole4-propionic acid receptor GluR1 subunit by calcium/calmodulin-dependent kinase II. J. Biol. Chem. *272,* 32528–32533.

McGee, A.W., and Bredt, D.S. (1999). Identification of an intramolecular interaction between the SH3 and guanylate kinase domains of PSD-95. J. Biol. Chem. *274,* 17431–17436.

Migaud, M., Charlesworth, P., Dempster, M., Webster, L.C., Watabe, A.M., Makhinson, M., He, Y., Ramsay, M.F., Morris, R.G., Morrison, J.H., et al. (1998). Enhanced long-term potentiation and impaired learning in mice with mutant postsynaptic density-95 protein. Nature *396,* 433–439.

Muller, B.M., Kistner, U., Kindler, S., Chung, W.J., Kuhlendahl, S., Fenster, S.D., Lau, L.-F., Veh, R.W., Huganir, R.L., Gundelfinger, E.D., and Garner, C.C. (1996). SAP102, a novel postsynaptic protein that interacts with NMDA receptor complexes in vivo. Neuron *17,* 255–265.

O'Brien, R.J., Lau, L.F., and Huganir, R.L. (1998). Molecular mechanisms of glutamate receptor clustering at excitatory synapses. Curr. Opin. Neurobiol. *8,* 364–369.

Pawson, T., and Scott, J.D. (1997). Signaling through scaffold, anchoring, and adaptor proteins. Science *278,* 2075–2080.

Roche, K.W., O'Brien, R.J., Mammen, A.L., Bernhardt, J., and Huganir, R.L. (1996). Characterization of multiple phosphorylation sites on the AMPA receptor GluR1 subunit. Neuron *16,* 1179–1188.

Rosenmund, C., Carr, D.W., Bergeson, S.E., Nilaver, G., Scott, J.D., and Westbrook, G.L. (1994). Anchoring of protein kinase A is required for modulation of AMPA/kainate receptors on hippocampal neurons. Nature *368,* 853–856.

Rubino, H.M., Dammerman, M., Shafit-Sagardo, B., and Erlichman, J. (1989). Localization and characterization of the binding site for the regulatory subunit of type II cAMP-dependent protein kinase on MAP2. Neuron *3,* 631–638.

Sheng, M., and Pak, D.T. (1999). Glutamate receptor anchoring proteins and the molecular organization of excitatory synapses. Ann. N Y Acad. Sci. *868,* 483–493.

Sheng, M., and Wyszynski, M. (1997). Ion channel targeting in neurons. Bioessays *19,* 847–853.

Soderling, T.R., and Derkach, V.A. (2000). Postsynaptic protein phosphorylation and LTP. Trends Neurosci. *23,* 75–80.

Sommer, B., Keinanen, K., Verdoorn, T.A., Wisden, W., Burnashev, N., Herb, A., Kohler, M., Takagi, T., Sakmann, B., and Seeburg, P.H. (1990). Flip and flop: a cell-specific functional switch in glutamate-operated channels of the CNS. Science *249,* 1580–1585.

Srivastava, S., Osten, P., Vilim, F.S., Khatri, L., Inman, G., States, B., Daly, C., DeSouza, S., Abagyan, R., Valtschanoff, J.G., et al. (1998). Novel anchorage of GluR2/3 to the postsynaptic density by the AMPA receptor-binding protein ABP. Neuron *21,* 581–591.

Swope, S.L., Moss, S.I., Raymond, L.A., and Huganir, R.L. (1999). Regulation of ligand-gated ion channels by protein phosphorylation. Adv. Second Messenger Phosphoprotein Res. *33,* 49–78.

Vallee, R.B., DiBartolomeis, M.J., and Theurkauf, W.E. (1981). A protein kinase bound to the projection portion of MAP 2 (microtubule-associated protein 2). J. Cell Biol. *90,* 568–576.

Wang, L.Y., Salter, M.W., and MacDonald, J.F. (1991). Regulation of kainate receptors by cAMP-dependent protein kinase and phosphatases. Science *253,* 1132–1134.

Wenthold, R.J., Yokotani, N., Doi, K., and Wada, K. (1992). Immunochemical characterization of the non-NMDA glutamate receptor using subunit-specific antibodies. Evidence for a hetero-oligomeric structure in rat brain. J. Biol. Chem. *267,* 501–507.

Westphal, R.S., Tavalin, S.J., Lin, J.W., Alto, N.M., Fraser, I.D., Langeberg, L.K., Sheng, M., and Scott, J.D. (1999). Regulation of NMDA receptors by an associated phosphatase-kinase signaling complex. Science *285,* 93–96.

Woods, D.F., Hough, C., Peel, D., Callaini, G., and Bryant, P.J. (1996). Dlg protein is required for junction structure, cell polarity, and proliferation control in Drosophila epithelia. J. Cell Biol. *134,* 1469–1482.

Wyszynski, M., Valtschanoff, J.G., Naisbitt, S., Dunah, A.W., Kim, E., Standaert, D.G., Weinberg, R., and Sheng, M. (1999). Association of AMPA receptors with a subset of glutamate receptor-interacting protein in vivo. J. Neurosci. *19,* 6528–6537.

Xia, J., Zhang, X., Staudinger, J., and Huganir, R.L. (1999). Clustering of AMPA receptors by the synaptic PDZ domain-containing protein PICK1. Neuron *22,* 179–187.

Yan, Z., Hsieh-Wilson, L., Feng, J., Tomizawa, K., Allen, P.B., Fienberg, A.A., Nairn, A.C., and Greengard, P. (1999). Protein phosphatase 1 modulation of neostriatal AMPA channels: regulation by DARPP-32 and spinophilin. Nat. Neurosci. *2,* 13–17.

Ziff, E.B. (1997). Enlightening the postsynaptic density. Neuron *19,* 1163–1174.