

SYNAPTIC AND SUBCELLULAR LOCALIZATION OF A-KINASE ANCHORING PROTEIN 150 IN RAT HIPPOCAMPAL CA1 PYRAMIDAL CELLS: CO-LOCALIZATION WITH EXCITATORY SYNAPTIC MARKERS

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Abstract—Excitatory and inhibitory ionotropic receptors are regulated by protein kinases and phosphatases, which are localized to specific subcellular locations by one of several anchoring proteins. One of these is the A-kinase anchoring protein (AKAP150), which confers spatial specificity to protein kinase A and protein phosphatase 2B in the rat brain. The distribution of AKAP150 was examined at rat hippocampal CA1 pyramidal cell asymmetric and symmetric post-synaptic densities and with respect to the distribution of markers of excitatory (vesicular glutamate transporter 1, glutamate receptor subunit 1) and inhibitory receptors (vesicular GABA transporter, GABA receptor type A β 2/3 subunits, gephyrin) and the Golgi marker, trans-Golgi network glycoprotein 38. AKAP150 was close to asymmetric synapses, consistent with numerous molecular and biochemical studies suggesting its interaction with components of the excitatory postsynaptic density. In contrast, we did not find AKAP150-immunoreactivity associated with inhibitory synapses in rat CA1 neurons, despite reports demonstrating an *in vitro* interaction between AKAP150 and GABA receptor type A receptor β subunits, and the reported co-localization of these proteins in rat hippocampal cultures. There was some overlap between AKAP150 and GABA receptor type A receptor β 2/3-immunoreactivity intracellularly in perinuclear clusters. These findings support previous work indicating the integration of kinase and phosphatase activity at excitatory synapses by AKAP150, but do not support a role for selective targeting of AKAP150 and its accompanying proteins to inhibitory synapses. © 2005 Published by Elsevier Ltd on behalf of IBRO.

Key words: protein kinase A, calcineurin, post-synaptic density, GABA_A receptors, receptor phosphorylation, receptor targeting.

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Abbreviations: AKAP, A-kinase anchoring protein; AKAP150-IR, A-kinase anchoring protein 150 immunoreactivity; EM, electron microscopic; GABA_A, GABA receptor type A; GABARAP, GABA_A receptor associated protein; GluR1-4, glutamate receptor subunits 1-4; LM, light microscopic; LTP, long term potentiation; PKA, protein kinase A; PKC, protein kinase C; PP2B, protein phosphatase type B; PSD, post-synaptic density; RACK1, receptor for activated C-kinase-1; SO, stratum oriens; SP, stratum pyramidale; SR, stratum radiatum; TGN38, trans-Golgi network glycoprotein 38; TMB, Tris–maleate buffer; VGAT, vesicular GABA transporter; VGLUT1, vesicular glutamate transporter 1.

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A-kinase anchoring proteins (AKAPs) are a group of functionally related proteins that bind the regulatory subunits of protein kinase A (PKA), and target the holoenzyme to discrete subcellular locations (Colledge and Scott, 1999; Diviani and Scott, 2001). The various AKAP isoforms can be discerned by their specific molecular interactions and subcellular distribution (Colledge and Scott, 1999; Edwards and Scott, 2000). AKAP150 (human AKAP79) interacts with the regulatory (RII β) subunit of PKA, protein kinase C (PKC), and protein phosphatase type B (PP2B) (calcineurin). Moreover, this AKAP isoform is believed to target its associated proteins to neuronal membranes and synapses, through domains capable of interacting with post-synaptic scaffold proteins including PSD-95, SAP-97, and F-actin, as well as the intracellular loops of GABA receptor type A (GABA_A) receptor β subunits (Carr et al., 1992; Colledge et al., 2000; Gomez et al., 2002; Brandon et al., 2003). Thus AKAP150 may confer spatial specificity to serine/threonine kinases and phosphatases at synaptic sites *in situ*.

Studies in recombinant and native neuronal systems have indicated that the cytoplasmic tails of glutamate receptor subunits (GluR)1, GluR2, and GluR4 AMPA receptor subunits are substrates for PKA, PKC, and CaMKII (Roche et al., 1996; Barria et al., 1997; McDonald et al., 2001). PKA modulates AMPA receptor currents via phosphorylation of GluR1-containing receptors at Ser845 (Greengard et al., 1991; Roche et al., 1996; Banke et al., 2000). In the hippocampus, PKA-mediated phosphorylation of Ser⁸⁴⁵ is permissive for synaptic incorporation of AMPA receptors during long term potentiation (LTP) induction and required for LTP maintenance (Nayak et al., 1998; Lee et al., 2000; Esteban et al., 2003). In addition, it is the AKAP150-anchored PKA and PP2B that are responsible for the phosphorylation and dephosphorylation of Ser845 on GluR1 (Tavalin et al., 2002).

Similarly, GABA_A receptors are regulated by protein phosphorylation (Swope et al., 1999; Olsen and Macdonald, 2002; Brandon et al., 2002). The cytoplasmic loops of GABA_A receptor β 1–3 subunits contain consensus PKA substrate sequences (Moss et al., 1992; McDonald and Moss, 1994), and increases or decreases in GABA_A receptor function have been reported depending on the β subunit residue phosphorylated (McDonald et al., 1998; Nusser et al., 1999; Poisbeau et al., 1999; Hinkle and Macdonald, 2003). The effects of phosphorylation on GABA_A receptor function may be mediated by PKA tethered to the receptor by AKAP150. Indeed, AKAP150 and GABA_A receptor β subunits co-immunoprecipitate from

whole rat brain lysates, and co-localize in rat hippocampal culture (Brandon et al., 2003).

Modulation of receptor trafficking and function via receptor phosphorylation could occur locally at synapses or at a distance from synaptic sites, likely directed by the precise subcellular localization of kinase/phosphatase anchoring complexes. Unfortunately the exact location of AKAP150 in neurons is uncertain. In one electron microscopy study (Sik et al., 2000) AKAP 79, the human ortholog of rodent AKAP150, was found postsynaptically in proximity to excitatory (asymmetrical) synapses in the human hippocampus. However, a light microscopy study (Glantz et al., 1992) failed to reveal AKAP150 immunoreactivity associated with excitatory synapses in rat hippocampal neurons. Moreover, despite the wealth of biochemical information relating AKAP150 to GABA_A receptor phosphorylation, neither of these studies found AKAP150 immunoreactivity at, or in close proximity to, inhibitory synapses. Therefore in this study, the distribution of AKAP150 was assessed in rat CA1 neuron synapses using both preembedding immunohistochemistry and dual immunofluorescence with markers of excitatory (vesicular glutamate transporter 1 (VGLUT1), GluR1) and inhibitory (vesicular GABA transporter (VGAT), GABA_A receptor β 2/3 subunits, gephyrin) synapses.

EXPERIMENTAL PROCEDURES

Tissue preparation

Eight adult male Sprague–Dawley rats (250–500 g, Harlan, Indianapolis, IN, USA), were perfused under anesthesia (120 mg/kg Na pentobarbital, i.p.) via the left ventricle with 0.9% NaCl containing 4% sucrose followed by 4% paraformaldehyde in 0.1 M PB (pH 7.4) with or without 0.25% glutaraldehyde for light (LM) and electron microscopic (EM) studies, respectively. For LM studies, blocks of tissue containing the hippocampus were post-fixed (1–6 h at 4 °C), equilibrated in 15% sucrose in PB, then frozen-sectioned in the coronal or sagittal plane at 40 μ m on a sliding microtome. Tissue intended for EM studies was blocked and sectioned at 50 μ m on a vibratome[®] (Pelco101, Ted Pella Inc., Redding, CA, USA). All sections were collected in PBS (0.01 M, pH 7.4) and stored at 4 °C until further processing.

Immunohistochemistry

Antibodies. The following antibodies were used: goat anti-AKAP150 (N-19, Santa Cruz, Santa Cruz, CA, USA), rabbit anti-GluR1 (R. Wenthold NIDCD, Bethesda, MD, USA), mouse anti-gephyrin (mAb 7a, Boehringer Mannheim, Indianapolis, IN, USA), guinea-pig anti-VGLUT1 (Chemicon, Temecula, CA, USA), mouse anti-GABA_A receptor β 2/3 (mAb341, bd-17, Chemicon), rabbit anti-VGAT (Chemicon), and mouse-anti-TGN-38 (Affinity Bioreagents, Golden, CO, USA).

LM immunohistochemistry. Free-floating sagittal sections (40 μ m) were slide-mounted (Colorfrost[®]/Plus slides, Fisher Scientific, Pittsburgh, PA, USA), blocked for 30 min in 10% normal horse serum diluted in PBS-T (0.01 M PBS+0.1% Triton X-100), and incubated overnight at 4 °C in anti-AKAP150 (1:200), rabbit anti-GluR1 (1:1000, 1 μ g/ml) or mouse anti-GABA_A receptor β 2/3 (1:100, 10 μ g/ml). After rinsing (3 \times 5 min, 1 \times 10 min PBS-T) tissues were exposed to HRP-conjugated biotin-sp-IgG raised in donkey, against goat (AKAP150), rabbit (GluR1), or mouse (GABA_A receptor β 2/3). Sections were rinsed, developed 5–10

min with a nickel-enhanced DAB (diaminobenzidine) reaction (Vector Laboratories, Burlingame, CA, USA), dehydrated and cleared with xylene, then coverslipped with Permount[™] (Fisher). Slide-mounted sections were visualized on a lightbox (Imaging Research Inc., St. Catherine, Ontario, Canada) under constant illumination and images were acquired with a high resolution CCD camera (Sierra Scientific, Sunnyvale, CA, USA) using NIH image (v. 1.59) software.

Double immunofluorescence. After 30 min in 10% normal horse serum diluted in PBS-T (0.01 M PBS+0.1% Triton X-100), free-floating sections were incubated overnight at 4 °C in anti-AKAP150 (1:100–1:200) alone or in combination with one of the following: rabbit anti-GluR1 (1:1000), mouse anti-gephyrin (1:100), guinea-pig VGLUT1 (1:4000), mouse anti-GABA_A receptor β 2/3 (1:100), rabbit anti-VGAT (1:200), or mouse anti-trans-Golgi network glycoprotein 38 (TGN38) (1:1000). After rinsing (3 \times 5 min, 1 \times 10 min PBS-T) tissues were exposed to Alexa anti-goat-568 (1:200, Molecular Probes, Eugene, OR, USA) alone or in combination with the appropriate secondary antibody: Alexa anti-mouse-488 (1:200, Molecular Probes), Alexa anti-rabbit-488 (1:200, Molecular Probes), or anti-guinea-pig-FITC antibodies (1:50, Jackson Laboratories). Sections were then rinsed and mounted on glass slides coated with 0.5% gelatin/0.05% chrome alum and coverslipped with Vectashield (Vector). Immunoreactivities were analyzed with an Olympus FX confocal microscope. High magnification was achieved using a Plan APO Olympus 60 \times objective lens (1.4 numerical aperture, theoretical resolution 0.17 μ m). Cross-talk between the emission of different fluorochromes at 488 nm with confocal microscopy was minimized as previously described (Geiman et al., 2002). Controls included the omission of the primary or secondary antibody in both single- and double-labeling experiments. These controls verified lack of significant auto-fluorescence, lack of secondary antibodies' interactions with naïve tissue, and the lack of secondary antibody interactions with primary antibodies of different species. In addition, AKAP150 antiserum specificity was tested by pre-absorption with the 19-amino acid immunizing peptide (20 μ g antiserum and 20 μ g peptide for 60 min at room temperature), before being applied to tissue sections. No staining was observed following preabsorption of the primary AKAP150 antiserum.

Preembedding immunohistochemistry. Vibratome sections (50 μ m) were exposed to 1% sodium borohydride in PBS for 30 min, rinsed 6 \times 5 min in PBS, and incubated in 10% normal chicken serum (Vector) for 3 h. Sections were then incubated in goat anti-AKAP150 (1:50) overnight with agitation at 4 °C. Tissues were rinsed (3 \times 5 min; 1 \times 10 min) and incubated for 3 h at room temperature in anti-goat-biotin (Vector), rinsed (3 \times 5 min; 1 \times 10 min) and exposed to avidin–biotin–peroxidase complex (Vector) for 2 h. After rinsing, the immunoreactive sites were visualized with DAB (0.005% a DAB reaction with 0.01% H₂O₂), rinsed again, fixed for 60 min in 2.5% glutaraldehyde, and washed in 0.1 M Tris–maleate buffer (TMB, pH 7.4) overnight. Sections were then silver-intensified with a silver nitrate solution (6 min), rinsed in TMB (3 \times 5 min), treated with 0.05% gold chloride (5 min), rinsed, incubated with 2.5% sodium thiosulfate (2 min), rinsed in TMB, followed by PBS, then fixed with 0.5% osmium tetroxide for 20 min. To prepare for embedding, sections were dehydrated through an ascending series of ethanols (50%, 2 \times 70%, 2 \times 95%, 3 \times 100%, 10 min each), propylene oxide (2 \times 10 min), 50% epon/araldite resin in propylene oxide (overnight) and then 100% resin (6 h). Tissues were flat-embedded in between glass coverslips were coated with formen-trenmittel (Electron Microscopy Sciences, Ft. Washington, PA, USA) and the resin was polymerized at 55 °C for 3 days. Areas of CA1 were excised from cured resin, mounted in EM capsules for recutting, ultrathin sections obtained and collected on nickel grids. The sections were analyzed in a Philips 201 electron microscope. Digital images were obtained using a Bio-

scan camera (Gatan Inc., Warrendale, PA, USA). Sections were scanned at low power in the electron microscope. When reaction product was observed, it was examined at higher power and its relationship to subcellular structures was recorded. Although peroxidase immunohistochemistry is not quantitative, this method provided a qualitative assessment of the subcellular distribution of AKAP150.

Colocalization analysis. Dual fluorescence colocalization was assessed using Image Pro Plus software v. 5.1 as previously described (Alvarez et al., 2004). At single confocal planes both colors in dual immunofluorescence images were superimposed, and 17 mm² regions of interest were analyzed from the dendritic (stratum oriens (SO) and stratum radiatum (SR)) and somatic (stratum pyramidale (SP)) layers of CA1 hippocampus. Each color immunofluorescence was thresholded to specifically detect immunoreactive objects. Threshold-segmented images for each color of immunofluorescence were superimposed and the number of objects showing co-localization compared with the total number of objects captured with each immunoreactivity. Immunoreactivities that showed less than 2% co-localization could arise because of

random partial overlap of close by structures, and were not considered significant.

RESULTS

Preliminary experiments with the anti-AKAP150 antiserum revealed that this protein is heterogeneously distributed in rat brain. A-kinase anchoring protein 150 immunoreactivity (AKAP150-IR) was most pronounced in the striatum and olfactory tubercle. The cortex and hippocampus expressed moderate levels, whereas midbrain and hindbrain were relatively devoid of AKAP150-IR (Fig. 1A). This pattern of expression was similar to that previously reported using a different antiserum against AKAP150 (Glantz et al., 1992). Moreover, all staining was eliminated when the antiserum was pre-absorbed with the immunizing peptide (Fig. 1C).

Within the hippocampus, AKAP150-IR was distributed over both neuronal and glial elements (Fig. 1B, D). The glial

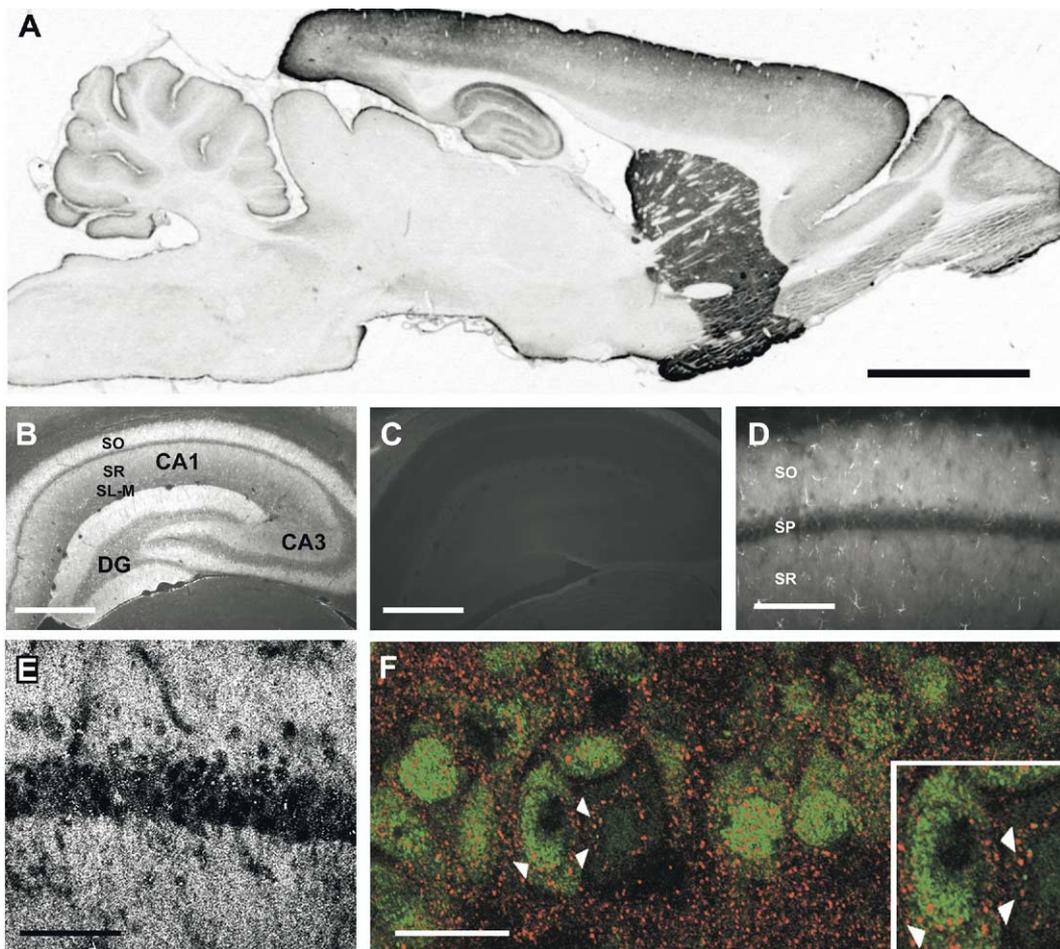


Fig. 1. AKAP150 immunohistochemistry. (A) Sagittal section of rat brain depicting AKAP150-IR. Note the relatively intense staining in the striatum and olfactory tubercle, as well as in some layers of both cortex and hippocampus. (B) AKAP150 is distributed throughout all but the most distal dendritic layers (stratum lacunosum-moleculare (SL-M)), and in the CA1 region stains basal dendrites (SO) more intensely than the apical dendrites (SR). (C) Pre-absorbing AKAP150 antiserum with the antigenic peptide eliminates the immunoreactivity. (D) CA1 of rat hippocampus. Glial immunoreactivity was more prominent at the tissue surface, and the staining became predominantly punctate at deeper levels (E). (F) High magnification of the CA1 pyramidal cell layer, demonstrating that AKAP150-IR (red) is arranged both at the cell periphery and in the cytoplasm, with clusters that surround the nucleus (arrowheads, inset enlarged 50%). In this panel an antibody against the nuclear antigen NeuN (green) demarcates nuclear and cell boundaries. Scale bars=5 mm (A), 1 mm (B, C), 200 μ m (D), 100 μ m (E) and 37.5 μ m (F).

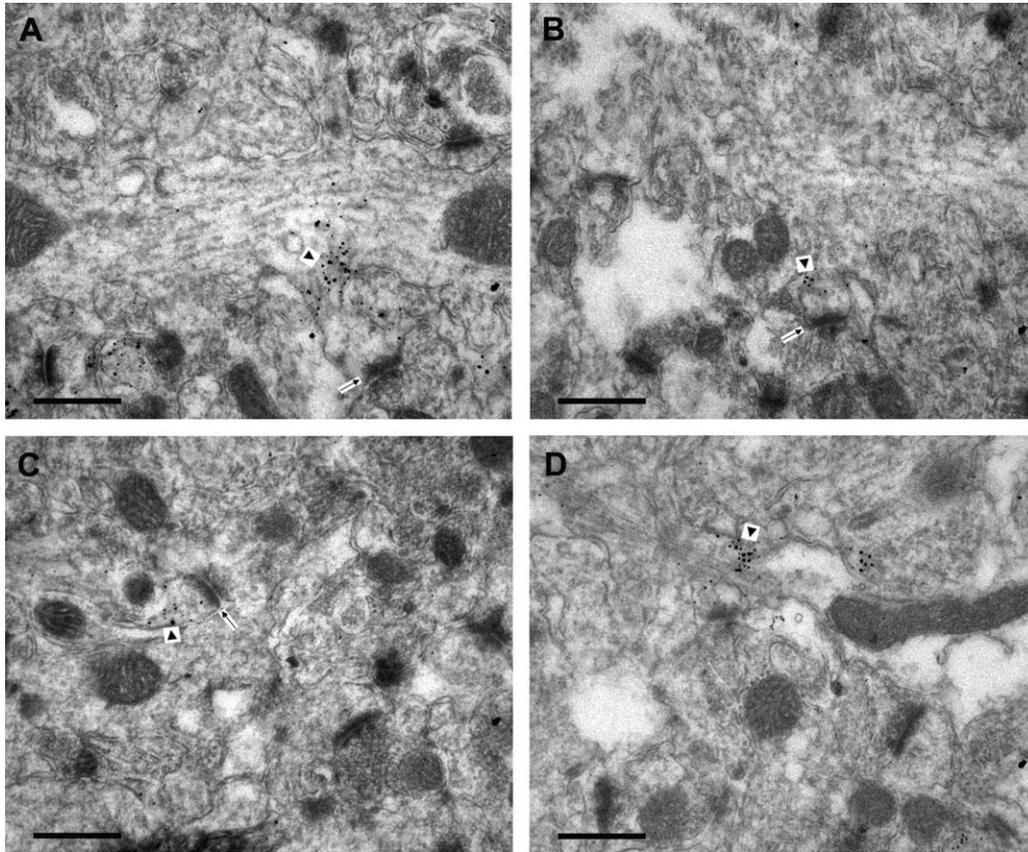


Fig. 2. Electron micrographs of AKAP150-IR in rat hippocampal CA1 neurons. Most immunoreactive profiles were found in proximity of asymmetric post-synapses. AKAP150-IR was concentrated in the neck of dendritic spines (reaction product denoted by arrowheads in A–C), proximate to post-synaptic densities (arrows in A–C). No immunoreactivity was observed in the vicinity of symmetric, presumed inhibitory, synapses. Immunoreactivity was also noted in glial elements, associated with glial filaments (arrowhead in D). Scale bars=0.5 μm .

staining was most obvious at the surface of the tissue section, and when shorter post-fixation was utilized. The significance of this staining is presently unclear. AKAP150-IR was not uniform in all regions. For example, CA1 and dentate gyrus showed stronger immunoreactivity than CA3. In addition, basal dendritic regions (SO) displayed greater immunoreactivity than did apical dendritic regions (SR; Fig. 1B). At higher magnification, AKAP150-IR was punctate (Fig. 1E), and when superimposed on NeuN-immunolabeled CA1 pyramidal cell somata revealed that most AKAP150-IR was located in the cytoplasm, while a smaller proportion of puncta were close to the cell surface (Fig. 1E, F).

The subcellular distribution of AKAP150 was further examined with electron microscopy and silver-intensified ABC peroxidase preembedding immunolabeling. AKAP150-IR appeared as clusters of silver particles over a weak diffuse DAB precipitate that frequently was not well resolved in metal-contrasted sections. Most AKAP150-IR was found in dendritic spines, usually in spine cytoplasm or the membrane region outside the PSD (post-synaptic density; Fig. 2A–C). Some AKAP150-IR was found in other dendritic regions and also on glial profiles (arrowheads, Fig. 2D). Although multiple CA1 layers were analyzed, there was no indication of AKAP150-IR in close association with symmetric, presumed inhibitory synapses. Control sec-

tions, including those from the thalamus (a region that does not express AKAP150) and those not exposed to AKAP antibodies showed no reaction product in neuronal or glial elements.

Although the ultrastructural studies indicated enrichment of AKAP150 in association with excitatory, but not inhibitory, synapses in CA1 of rat hippocampus, it was important to reevaluate these findings with light microscopy, where less stringent fixation and membrane permeabilization could be used, allowing for increased detection sensitivity. First, immunofluorescent labeling for AKAP150 was compared with markers of presynaptic (VGLUT1) or postsynaptic elements (GluR1) of excitatory synapses. Preliminary observations confirmed the robust hippocampal expression of VGLUT1 reported elsewhere (Fig. 3A; Herzog et al., 2001). AKAP150-IR did not overlap with VGLUT1 staining in any hippocampal layer suggesting that AKAP150 is not localized presynaptically. However, there were regions where VGLUT1-IR was apposed to AKAP150-IR in the cellular and dendritic layers of CA1 (arrowheads, Fig. 3B, C). GluR1 immunoreactivity was also strong in the rat hippocampus, and also reflected the pattern previously reported (Petralia and Wenthold, 1992) with a relatively dense distribution in the CA1 region (Fig. 3D). Although there was occasional overlap of AKAP150

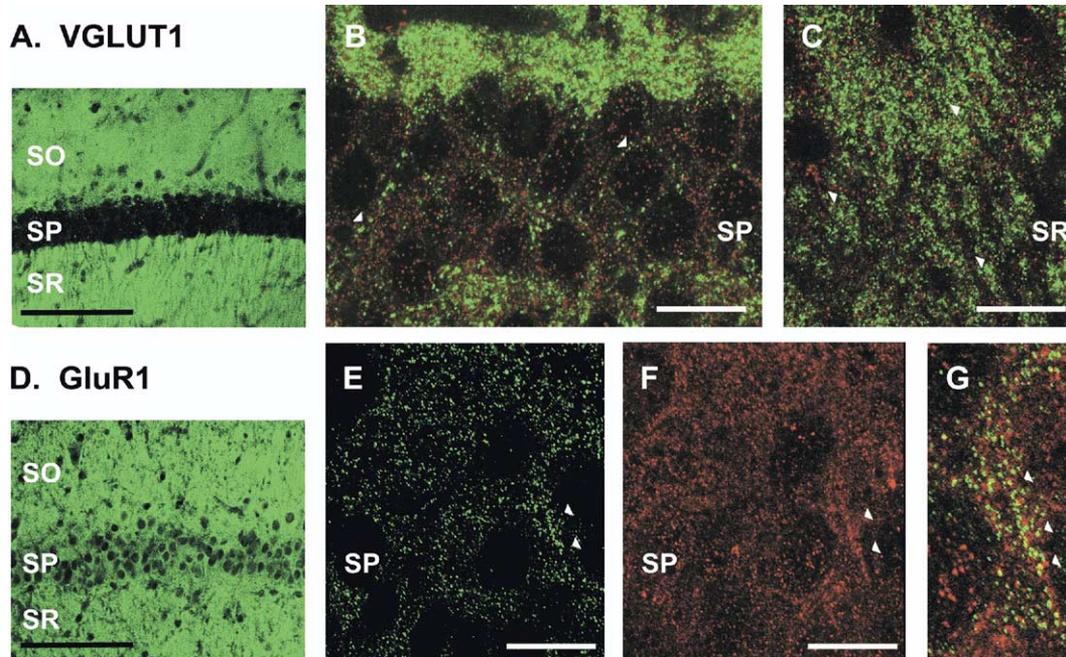


Fig. 3. Immunohistochemistry of AKAP150 and excitatory synaptic markers. (A) VGLUT1-IR in the CA1 region. (B) High magnification image of CA1 SP. Note that VGLUT1 (green) and AKAP150 (red) do not overlap, but are interspersed (see arrowheads) in both this layer, and in dendritic layers (SR, panel C). (D) GluR1-IR in the CA1 region. (E) High magnification image of GLUR1-IR in CA1 SP. Note the GluR1-IR (green) is present throughout the pyramidal cell cytoplasm, and partially overlaps with AKAP150 (arrowheads, lower right) (F), enlarged 200% in G (arrowheads). Scale bars=100 μm (A, D); 25 μm (B, C, E, F).

and GluR1, less than 1% of AKAP clusters were associated with GluR1 immunoreactivity (Fig. 3D–F). This is perhaps a reflection of the subsynaptic distribution of AKAP150 noted with electron microscopy.

To assess whether AKAP150 was present at rat hippocampal CA1 inhibitory synapses, dual immunofluorescence studies were carried out with AKAP150 and markers of inhibitory terminals (VGAT) and postsynaptic elements (gephyrin and GABA_A receptor β 2/3 subunit). VGAT is present in the synaptic vesicles of inhibitory terminals, and in the rat hippocampus clearly demarcates the cellular somatic layer of CA1 (Fig. 4A). VGAT immunoreactivity in CA1 somatic (Fig. 4B) or dendritic (Fig. 4C) layers did not co-localize with AKAP150-IR in these layers. Interneurons in CA1 dendritic layers often exhibited perinuclear AKAP clusters usually more prominent than those evident in pyramidal neurons. Gephyrin, a marker of inhibitory PSDs (Sassoè-Pognetto and Fritschy, 2000), likewise exhibited more pronounced staining in the somatic layer of CA1 (Fig. 4D). As the gephyrin antibody is more sensitive to fixation, a shorter post-fixation (1 h) was used to assess overlap. Glial staining for AKAP was appreciably greater in the lighter-fixed tissue. There was no significant enrichment of AKAP-immunoreactivity (i.e. <1% colocalization) associated with gephyrin clusters in either the cellular (Fig. 4E–G), or dendritic layers of CA1.

In hippocampal cell cultures co-localization between of AKAP150 and β 2/3 immunoreactivities have been reported (Brandon et al., 2003). We utilized a monoclonal antibody to GABA_A receptor β 2/3 subunits in conjunction with the antiserum to AKAP150 to explore this possible

interaction in intact hippocampus. Again, less than 1% of AKAP150 clusters were associated with β 2/3 immunoreactivity (Fig. 4J). Interestingly, the limited overlap detected was largely cytoplasmic and distributed in clusters around the nuclear periphery (Fig. 4J). Whether this overlap is functionally significant is presently unclear.

AKAP150-IR was clearly associated with intracellular structures surrounding the nucleus, where it co-localized with TGN38, a transport protein specifically located in the Golgi apparatus (Fig. 5A–C). In the CA1 region, 10% of AKAP150 clusters contained also TGN-38 immunoreactivity. This co-localization was most apparent in the prominent Golgi apparatus displayed by hilar interneurons (Fig. 5D–F).

DISCUSSION

This study extends previous studies that describe AKAP150 immunoreactivity in multiple layers of the hippocampal CA1 region and provides the first direct ultrastructural evidence that AKAP150 immunoreactivity is associated with excitatory synaptic profiles in rat hippocampal CA1 pyramidal cells, *in situ*. Using both EM and LM immunochemical techniques, we found no evidence for the presence of AKAP150 at inhibitory synapses in the rat hippocampus, suggesting that there is no anatomical basis for a stable interaction between AKAP150-anchored PKA and GABA_A receptor β subunits at the synapse. The identification of AKAP150 in clusters around the nucleus and in association with a marker of the Golgi apparatus offers the

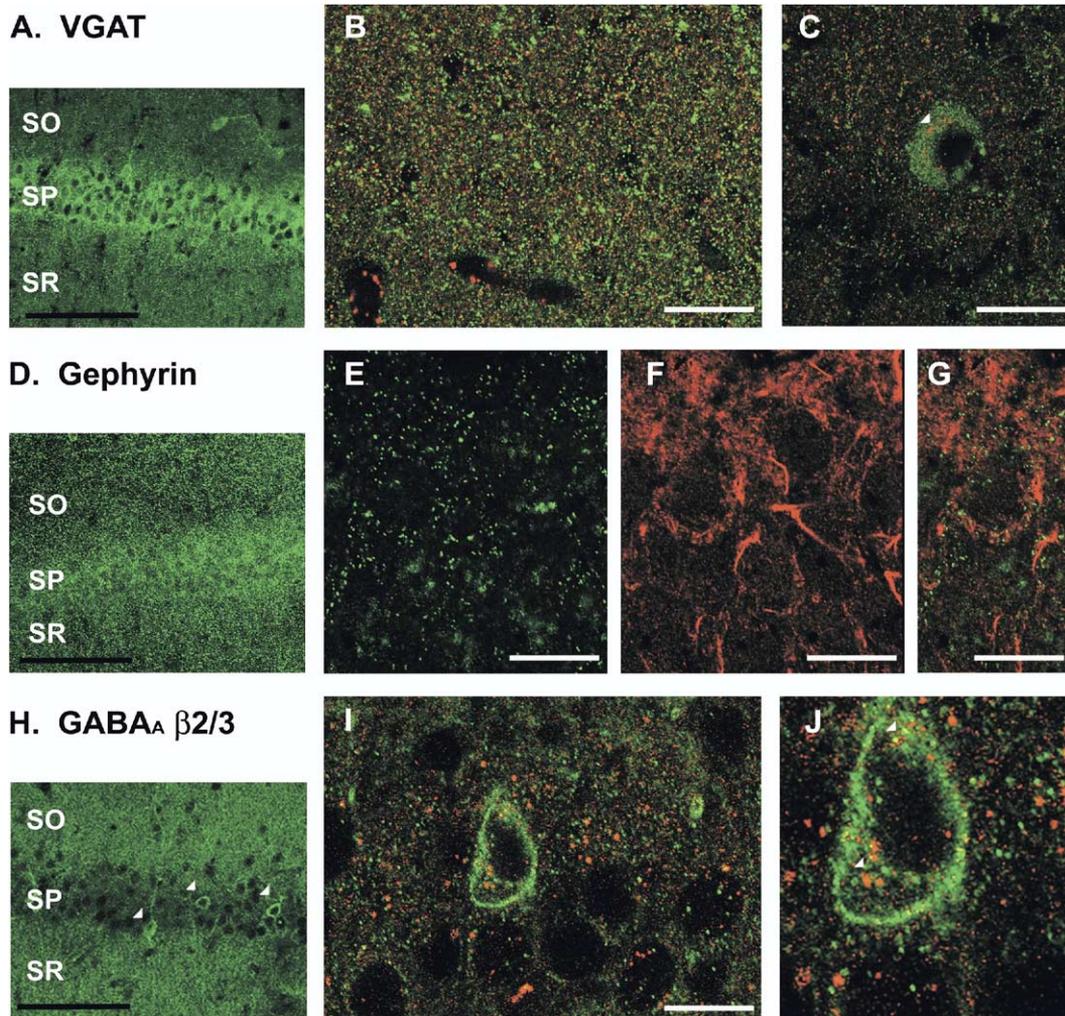


Fig. 4. Immunohistochemistry of AKAP150 with inhibitory synaptic markers. (A) VGAT-IR in the CA1 region. (B) High magnification photomicrograph of CA1 SP. Although the VGAT (green) and AKAP150 (red) clusters are interspersed, a common overlapping pattern is not easily discernable. (C) Image from SR depicting a VGAT immunoreactive interneuron, with AKAP-IR arranged around the nucleus (arrowhead). (D) Gephyrin-IR in the CA1 region. When higher magnification images of gephyrin (E) and AKAP150 (F) in SP of CA1 are overlaid (G), puncta representing these proteins do not co-localize. (H) GABA_A receptor β 2/3-IR in CA1 of hippocampus. Note the presence of numerous well-stained interneurons in SP. (I) High magnification image depicting β 2/3-IR (green) and AKAP150-IR (red) in the pyramidal cell layer of CA1. Panel I is enlarged 200% in J. Although limited, the overlap that is present between β 2/3 and AKAP150 is more obvious in interneurons, and present within cytoplasmic clusters (arrowheads). Scale bars=100 μ m (A, D, H); 25 μ m (B, C, E–G, I).

possibility that the AKAP150 plays a role in the trafficking of membrane-targeted proteins.

AKAP150 is present throughout rat hippocampal formation, but restricted to CA1 in human hippocampus. Notwithstanding these differences, here we report that the ultrastructural distribution of AKAP150 in the rat CA1 region is similar to the distribution of its ortholog in CA1 of human hippocampus (Sik et al., 2000). Our findings are consistent with multiple biochemical and molecular studies that support the association of AKAP150 and excitatory post-synapses (Bregman et al., 1989; Coghlan et al., 1995; Klauck et al., 1996; Colledge et al., 2000). Moreover, the findings provide a direct structural correlate for functional studies that implicate the PKA-AKAP150-PP2B complex in regulating short- and long-term changes in hippocampal

excitatory receptor function (Rosenmund et al., 1994; Abel et al., 1997; Tavalin et al., 2002).

Asymmetric synapses contain NMDA- and AMPA-type glutamate receptors embedded in a prominent membrane thickening composed of multiple proteins, the PSD (Gray, 1959; Kennedy, 1997; Walikonis et al., 2000). Scaffolding molecules, like those in the AKAP family, are proposed to play a role in recruiting signaling molecules to the postsynaptic membrane (Colledge and Scott, 1999; Edwards and Scott, 2000; Diviani and Scott, 2001). However, in the present immunofluorescence studies, most AKAP150 clusters were not associated with GluR1 clusters. Ultrastructural analysis revealed that AKAP150 immunoreactivity was clearly associated with dendritic spines, subjacent to the post-synapse, consistent with the lack of co-local-

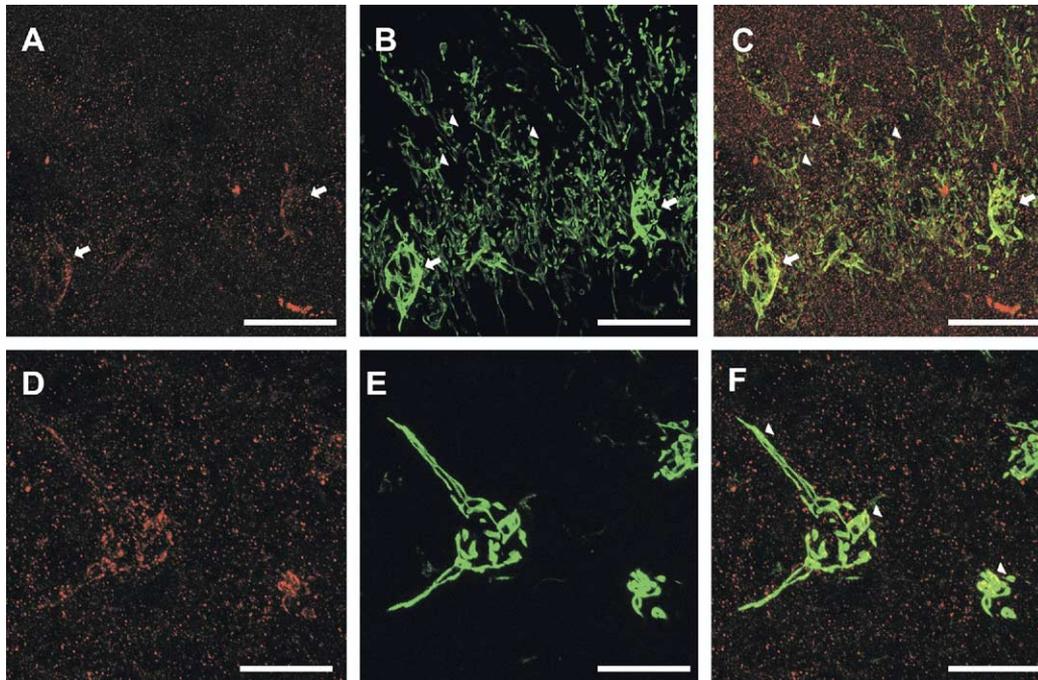


Fig. 5. (A) AKAP150-IR in the CA1 pyramidal cell layer of the hippocampus. (B) TGN38-IR at the same plane as in (A), depicting the Golgi apparatus of pyramidal cells (arrowheads) and interneurons (arrows). (C) When overlaid, there are multiple areas of colocalization over both pyramidal cells (arrowheads) and interneurons (arrows), although those on interneurons are more apparent. (D) AKAP150-IR in the hilus of the rat dentate gyrus. (E) TGN38-IR at the same plane as in (D), depicting the extensive Golgi apparatus in hilar interneurons. (F) When overlaid AKAP150 and TGN38 colocalize in these interneurons (arrowheads). Scale bars=25 μm .

ization between AKAP150 and GluR1. The immunoperoxidase label offers the advantage of greater sensitivity, but the reaction product tends to diffuse, and therefore lacks the spatial resolution of immunogold labeling. However, little or no immunoreactivity was detected in PSDs suggesting that diffusion of the reaction product from these structures is unlikely to have caused errant localization of AKAP150-immunoreactivity in the cytoplasm. Unfortunately we were unable to obtain adequate labeling using this antibody with post-embedding techniques. It is possible that AKAP150-immunoreactivity could have been masked by reduced antibody accessibility or steric hindrance with the macromolecular PSD complex. Using immunogold, Sik et al. (2000) also reported AKAP79 immunoreactivity near, but not within PSDs. Considering the similarity between the ultrastructural distribution of AKAP79/150 immunoreactivity in the human and rat hippocampal CA1 regions reported by Sik et al. (2000), and in this study using two different immunocytochemical EM methods and different antibodies, we conclude that AKAP150 may be preferentially located in cytoplasmic regions in proximity to excitatory synapses, but not within the PSD.

Molecular studies have indicated that AKAP150 is linked to the GluR1 subunit by the post-synaptic protein SAP97 (Colledge et al., 2000) and maintains AMPA receptor function by locally recruiting PKA (Rosenmund et al., 1994; Tavalin et al., 2002). In contrast, yotiao, an alternate AKAP isoform, directly interacts with the NR1 subunit of the NMDA receptor and active protein phosphatase 1,

suppressing NMDA receptor function by promoting NR1 dephosphorylation (Westphal et al., 1999; Edwards and Scott, 2000). Although PKA bound to AKAPs may be inactive (Faux et al., 1999; Edwards and Scott, 2000), disruption of the AKAP150-PKA interaction suppresses AMPA receptor function and phosphorylation on Ser845 (Rosenmund et al., 1994; Tavalin et al., 2002).

The subsynaptic localization of AKAP150 may allow for local sequestration of PKA, which upon dissociation from AKAP150 is catalytically active and required to maintain the phosphorylation of synaptic GluR1 subunits. When excitatory synaptic strength is increased following LTP induction, AKAP150 is upregulated (Genin et al., 2003), perhaps recruiting more PKA. This might increase excitatory synaptic function and receptor incorporation (Lee et al., 2000; Esteban et al., 2003).

There is ample evidence for PKA-mediated regulation of GABA_A receptors (McDonald et al., 1998; Poisbeau et al., 1999; Nusser et al., 1999; Olsen and Macdonald, 2002; Hinkle and Macdonald, 2003). Moreover, Brandon et al. (2003) reported an association between AKAP150 and GABA_A receptor β subunits, and co-localization of AKAP150 and β 2/3 in rat hippocampal culture. However, we did not find AKAP150 at inhibitory synapses in CA1 of rat hippocampus. This finding is consistent with the absence of AKAP79 at inhibitory synapses in the human hippocampus (Sik et al., 2000), and fails to provide a structural basis for local regulation by AKAP150-anchored PKA of GABA receptors at the synapse. Similarly, receptor for activated C-kinase-1 (RACK1) and PKC co-immuno-

precipitate with cortical GABA_A receptor β subunits, interactions that are necessary for the PKC-mediated attenuation of whole-cell GABA currents in cultured neurons (Brandon et al., 2002). However, neither PKC nor RACK1 have been identified immunohistochemically in association with inhibitory synapses in the CNS. Unlike excitatory synapses, inhibitory profiles do not exhibit a prominent PSD, are not associated with spines, and in the hippocampus are concentrated at the cell soma and proximal processes (Megias et al., 2001). In the absence of an anchoring protein, one possibility is that the receptors at inhibitory synapse PSDs are regulated by cytoplasmic changes in kinase and phosphatase activity, rather than being controlled by compartmentalized aggregates of regulatory enzymes, including PKA and PKC in the PSDs. Alternatively, post-translational modifications of synaptic GABA_A receptors may occur at the synapse through recruitment of kinases and phosphatases by scaffolding molecules yet to be identified.

The perinuclear staining of AKAP150 and its overlap with the Golgi-apparatus marker TGN38 is intriguing. A similar pattern of Golgi-staining was observed in a variety of cell lines with an alternate AKAP isoform, AKAP350. Notably, AKAP350 has multiple splice variants, including the NMDA receptor binding AKAP, yotiao (Schmidt et al., 1999). It is interesting to speculate that the Golgi- and receptor-associated AKAP variants regulate excitatory tone by affecting receptor trafficking and synaptic receptor function, following transcription of a single gene. The presence of AKAP150 immunoreactivity in perinuclear clusters and in association with TGN38 may indicate a Golgi-associated function for AKAP150. Like AKAP150, GABA_A receptor associated protein (GABARAP) binds to GABA_A receptor subunits *in vitro* and co-localizes with GABA_A receptors in neuronal culture. Additionally, GABARAP is found in the Golgi apparatus and now thought related to the intracellular trafficking of GABA_A receptors (Kneussel et al., 2000; Kittler et al., 2001). An alternate possibility is that AKAP150 is clustered at the Golgi-apparatus in transit to the sub-synapse, where it tethers local signaling molecules. Albeit limited, the co-localization between AKAP150 and the GABA_A receptor β 2/3 subunit in perinuclear clusters offers the possibility that the previously reported interaction between AKAP150 and GABA_A receptors (Brandon et al., 2002) in part occurs in the Golgi apparatus.

CONCLUSIONS

The present study indicates that AKAP150 immunoreactivity is present in proximity of excitatory synapses in rat hippocampal CA1 neurons. Together with previous functional studies pertaining to the effect of AKAP150 and its associated kinases on excitatory receptor function, these findings confirm the existence of an endogenous pool of AKAP150 in the proximity of synaptic excitatory ionotropic receptors. Conversely, we did not find AKAP150 immunoreactivity in association with inhibitory synapses in rat CA1 pyramidal cells.

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