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

S. M. LILLY,[a] F. J. ALVAREZ[b1] AND E. I. TIETZ[a1]*

[a]*Department of Pharmacology and the Cellular and Molecular Neurobiology Program, 3035 Arlington Avenue, Medical University of Ohio, Toledo, OH 43614, USA*

[b]*Department of Anatomy and Physiology, 3640 Colonel Glenn Highway, Wright State University, Dayton, OH 45435, USA*

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 β2/3 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 β2/3-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.

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 (RIIβ) 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 Ser^{845} 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 β1–3 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

[1] An equal contribution was made from the laboratories of F.J.A. and E.I.T.

*Corresponding author. Tel: +1-419-383-4170, fax: +1-419-383-2871.
E-mail address: etietz@mco.edu (E. Tietz).

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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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 β2/3 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 μm on a sliding microtome. Tissue intended for EM studies was blocked and sectioned at 50 μm 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 β2/3 (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 μm) 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 μg/ml) or mouse anti-$GABA_A$ receptor β2/3 (1:100, 10 μg/ml). After rinsing (3×5 min, 1×10 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 β2/3). 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 β2/3 (1:100), rabbit anti-VGAT (1:200), or mouse anti-trans-Golgi network glycoprotein 38 (TGN38) (1:1000). After rinsing (3×5 min, 1×10 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× objective lens (1.4 numerical aperture, theoretical resolution 0.17 μm). 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 μg antiserum and 20 μg 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 μm) were exposed to 1% sodium borohydride in PBS for 30 min, rinsed 6×5 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 (3×5 min; 1×10 min) and incubated for 3 h at room temperature in anti-goat-biotin (Vector), rinsed (3×5 min; 1×10 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% H_2O_2), 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 (3×5 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%, 2×70%, 2×95%, 3×100%, 10 min each), propylene oxide (2×10 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-