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PROTEIN INTERACTING WITH C KINASE 1 (PICK1) AND GLUR2 ARE ASSOCIATED WITH PRESYNAPTIC PLASMA MEMBRANE AND VESICLES IN HIPPOCAMPAL EXCITATORY SYNAPSES

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Abstract—AMPA receptors have been identified in different populations of presynaptic terminals and found to be involved in the modulation of neurotransmitter release. The mechanisms that govern the expression of presynaptic AMPA receptors are not known. One possibility is that pre- and postsynaptic AMPA receptors are regulated according to the same principles. To address this hypothesis we investigated whether protein interacting with C kinase 1 (PICK1), known to interact with AMPA receptors postsynaptically, also is expressed presynaptically, together with AMPA receptors. Subfractionation and high-resolution immunogold analyses of the rat hippocampus revealed that GluR2 and PICK1 are enriched postsynaptically, but also in presynaptic membrane compartments, including the active zone and vesicular membranes. PICK1 and GluR2 are associated with the same vesicles, which are immunopositive also for synaptophysin and vesicle-associated membrane protein 2. Based on what is known about the function of PICK1 postsynaptically, the present data suggest that PICK1 is involved in the regulation of presynaptic AMPA receptor trafficking and in determining the size of the AMPA receptor pool that modulates presynaptic glutamate release. © 2009 Published by Elsevier Ltd on behalf of IBRO.

Key words: hippocampus, synapse, AMPA receptors, PICK1, trafficking, vesicles.

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Abbreviations: ACy, astrocyte cytoplasm; AM, astrocyte membrane; AMPAR, AMPA receptor; AZ, active zone; BAR, Bin/amphiphysin/Rvs; BSA, bovine serum albumin; DCy, dendrite cytoplasm; DM, dendritic plasma membrane; EDTA, ethylenediaminetetraacetic acid; EM, electron microscopy; FA, formaldehyde; GA, glutaraldehyde; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IF, immunofluorescence; LM, light microscopy; NaPi, sodium phosphate buffer; NCS, normal calf serum; NMDA, *N*-methyl-D-aspartate; ON/RT, overnight at room temperature; PAG, phosphate-activated glutaminase; PICK1, protein interacting with C kinase; PoCy, postsynaptic cytoplasm; PoL, synaptic lateral membranes, i.e. on each side of the postsynaptic specialization; PreCy, presynaptic cytoplasm; PreL, synaptic lateral membranes, i.e. on each side of the active zone; PSS, postsynaptic specialization; SDS, sodium dodecyl sulfate; VAMP2, vesicle-associated membrane protein 2; VGAT, vesicular GABA transporter; WB, Western blot.

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Glutamate signaling occurs at a majority of the excitatory synapses in the brain, by acting on three different types of glutamate receptors: the ionotropic AMPA/kainate and *N*-methyl-D-aspartate (NMDA) receptors (Mayer, 2005), and the metabotropic receptors (Conn and Pin, 1997). AMPA receptors (AMPARs) are hetero-oligomeric complexes composed of four subunits, GluR1-4 (Rosenmund et al., 1998; Dingledine et al., 1999). In the hippocampus, the predominant subunit combinations are GluR1/GluR2 and GluR2/GluR3 (Wenthold et al., 1996).

Recent studies have shown that AMPAR subunits are trafficked between the postsynaptic plasma membrane and an intracellular membrane compartment (Contractor and Heinemann, 2002; Gerges et al., 2004; Steiner et al., 2005; Feligioni et al., 2006). This trafficking occurs constitutively and is also involved in synaptic plasticity like long term potentiation and long term depression (Malenka, 2003; Kakegawa and Yuzaki, 2005). The AMPAR recycling depends on interactions between AMPAR subunits and a number of cytosolic proteins. One of these proteins, protein interacting with C kinase 1 (PICK1) (Dev et al., 1999; Xia et al., 1999; Rocca et al., 2008), has been shown to interact with the GluR2 subunit at postsynaptic sites and to be involved in NMDA-induced internalization of AMPARs from the plasma membrane.

Hanley and Henley (2005) have shown that PICK1 has a Ca^{2+}-binding site that is engaged in the regulation of GluR2–PICK1 interaction under different physiological ranges of Ca^{2+} concentrations. Overexpression in hippocampal neurons of a PICK1 mutant that binds strongly and Ca^{2+} independently to GluR2 occludes NMDA-induced AMPAR endocytosis (Hanley and Henley, 2005). The finding (Peter et al., 2004) that PICK1 belongs to a large family of proteins containing a Bin/amphiphysin/Rvs (BAR) domain supports a direct role for PICK1 in endocytosis (Lu and Ziff, 2005; Jin et al., 2006). BAR domains contain crescent-shaped dimers that bind preferentially to curved, negatively charged membranes. Postsynaptic colocalization of PICK1 and GluR2 has been observed at the light microscopic level (Chung et al., 2000), but this finding has not yet been validated by electron microscopy (EM). It has been suggested that AMPARs may mediate also presynaptic effects (Satake et al., 2000; Takago et al., 2005; Schenk et al., 2005), in glutamatergic as well as GABAergic terminals and in growth cones. Thus, a novel role for AMPARs as modulators of presynaptic structure and function is emerging. However, the mechanisms governing trafficking of presynaptic AMPARs have not been resolved. Some reports have suggested that presynaptic AMPARs

modulate neurotransmitter release in selected synapses (Satake et al., 2000; Takago et al., 2005; Pittaluga et al., 2006). Furthermore, synaptosome studies indicate that PICK1 is involved in the regulation of presynaptic GluR2 subunits (Pittaluga et al., 2006). The aim of the present study was to resolve whether PICK1 and GluR2 are coexpressed in the presynaptic compartment of hippocampal excitatory synapses, in order to substantiate the concept that PICK1 is involved in the regulation of presynaptic AMPARs.

EXPERIMENTAL PROCEDURES

Antibodies

The following polyclonal antibodies were used: PICK1 (1:300 for light microscopy (LM) and immunofluorescence (IF), 1:100 for EM, 1:5000 for Western blots (WB); Abcam, Cambridge, UK); GluR2 (LM 1:300, EM 1:30, WB 1:2000; Chemicon, Temecula, CA, USA), GluR2/3 (WB 1:2000; Chemicon), and phosphate-activated glutaminase (PAG) (WB 1:200,000; gift from Dr. B. Roberg, University of Oslo, Oslo, Norway) (Laake et al., 1999). Monoclonal antibodies used were GluR2 (IF 1:300; Chemicon), synaptophysin (IF 1:300; WB 1:2000; Chemicon), PSD95 (WB 1:2000; Abcam), vesicle-associated membrane protein 2 (VAMP2) (WB 1:2000; Covance, Princeton, NJ, USA), anti–glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (WB 1:10,000, Abcam); vesicular GABA transporter (VGAT) (IF 1:3000, Chemicon). The secondary antibodies used were: anti-mouse Alexa Fluor 488 (IF 1:1000; Molecular Probes, Eugene, USA), anti-rabbit Cy3 (IF 1:1000; Jackson Immuno, Baltimore, USA), anti-mouse FITC monovalent Fab fragments (IF 1:100; Jackson Immuno), and IgG coupled to 10 or 6 nm colloidal gold (EM 1:40; British BioCell International, Cardiff, UK).

Animals

Wistar male rats weighing 250–300 g (Møllegaard, Ejby, Denmark) were used for this study. Experimental protocols were approved by the Institutional Animal Care and Use Committee and conform to National Institutes of Health guidelines for the care and use of animals, as well as international laws on protection of laboratory animals, with the approval of a local bioethical committee and under the supervision of a veterinary commission for animal care and comfort of the University of Oslo. Every effort was made to minimize the number of animals used and their sufferings.

Transfection of cell cultures

Primary hippocampal cultures containing both neurons and glial cells of 1–5 day old rats (Wistar) were prepared as previously described (Vik-Mo et al., 2003). They were transfected at 4 days *in vitro* (DIV). Transfection was performed using 0.24 μg siRNA (Stealth, Invitrogen) and 0.6 μl Lipofectamine 2000 (Invitrogen) per well and thus a ratio 2.5:1 of Lipofectamine 2000 to siRNA (volume to weight) was used. Complexation time was 30 min and total volume added per well was 500 μl. The transfection time was 4 h after which the transfection mixture was replaced by the original medium. Cells were harvested at either 24 or 48 h after transfection by adding 1% sodium dodecyl sulfate (SDS) in 10 mM sodium phosphate buffer (NaPi) on a shaker for 30 min. As control group (MOCK) cells were treated in the same way without siRNA. The homogenates were run on 10% SDS–acrylamide (Bio-Rad), electroblotted onto nitrocellulose membrane (Hoefer Scientific Instruments, San Francisco, CA, USA) and immunostained with primary antibodies against PICK1 and GAPDH and horseradish peroxidase–linked secondary antibody (Amersham Biosciences, UK). The signal was detected by enhanced chemiluminescence using Supersignal westpico chemiluminescence substrate (Pierce). The chemiluminescence signals were visualized by a chemiluminescence digital camera detection system (Fuji LAS 3000), and quantified by using ImageQuant TL (Amersham Biosciences). The ratio between PICK1 and GAPDH signal was obtained. The level of GAPDH was used as an indicator of the number of cells per well.

HeLa cell cultures were grown in DMEM with 10% fetal calf serum (Gibco BRL, Invitrogen) and 0.1% glutamine. The cells were plated at 80% confluence 24 h before transfection. Transfection of full-length rat PICK1 construct (Open Biosystems, AL, USA) was performed by complexing 0.2 μg of plasmid DNA with 0.6 μg Lipofectamine 2000 (Invitrogen) per well, in triplicate, for 30 min in a volume of 200 μl serum free medium. After complexation, the DNA:Lipofectamine 2000 mix was diluted to 500 μl per well using normal medium, and added to wells. After 4 h of transfection, the transfection medium was replaced with normal medium. The cells were incubated further for 24 h and then fixed in 4% formaldehyde (FA) for 30 min.

Immunocytochemistry

Perfusion fixation. For EM studies the rats (n=3) were deeply anesthetized with Equithesin (0.4 ml/100 g body weight) followed by intracardiac perfusion with 10–15 s flush of 4% Dextran-T70 in NaPi (pH 7.4) followed by a mixture of 4% FA and 0.1% glutaraldehyde (GA) in the same buffer. For LM, the perfusion fixative was 4% FA.

Bright field microscopic studies. Free floating Vibratome sections from rat brain (50 μm) were treated with 1 M ethanolamine-HCl (pH 7.4), blocked with 3% (v/v) normal calf serum (NCS) in 0.1 M NaPi pH7.4, and incubated with antibodies against PICK1 or GluR2, overnight at room temperature (ON/RT), followed by incubation with secondary antibodies for 1 h at RT and development with the biotin–streptavidin–peroxidase system and 3,3′-diaminobenzidine (DAB) (Sigma).

Confocal laser scanning microscopy studies. The tissue was prepared as described above, except that the sections were double labeled with the anti-PICK1 and one of the monoclonal antibodies in 3% (v/v) NCS and 1% (w/v) bovine serum albumin (BSA; Sigma) in NaPi (ON/RT). The sections were rinsed in NaPi, incubated for 2 h with secondary antibodies at RT and rinsed again in NaPi. IF double labeling with antibodies from the same host species was performed by fixing the sections again with 4% FA for 45 min, after incubation with the first secondary antibodies (FITC monovalent Fab-fragments). The sections were rinsed with NaPi, blocked, and incubated with the second primary antibody (ON/RT). The following steps were done as described above. The tissue sections were mounted with Prolong mounting media (Molecular Probes), and examined with an Axioplan 2 equipped with a LSM 5 Pascal scanner head (Carl Zeiss, Heidelberg, Germany).

EM studies. Small (0.5×1.0 mm) blocks from CA1 were freeze substituted, sectioned, and immunolabeled essentially as described previously (Mathiisen et al., 2006). The sections were examined with Philips CM 10 or Fei Tecnai 12 electron microscopes at 60 kV. Pre-adsorption with the immunizing peptide and omission of the primary antibody were used as negative control for immunogold cytochemistry.

EM quantification and statistical analysis. Electron micrographs were obtained at random from the middle layer of stratum radiatum of the CA1 region of the hippocampus. PICK1 immunolabeling was quantified as number of gold particles/μm of membrane length in asymmetric synapses or as number of gold particles/μm^2 for regions of interests in the intracellular compartment. Specific plasma membrane and cytoplasmic compartments were defined and used for quantifications. They correspond to the