Neuroscience 143 (2006) 953–964

HUMAN AUTOANTIBODIES AGAINST EARLY ENDOSOME ANTIGEN-1 ENHANCE EXCITATORY SYNAPTIC TRANSMISSION

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Abstract—Early endosome antigen 1 (EEA1), a peripheral membrane protein associated with the cytoplasmic face of early endosomes, controls vesicle fusion during endocytosis, as extensively studied in non-neuronal cells. In neurons, early endosomes are involved in recycling of synaptic vesicles and neurotransmitter receptors. Since certain patients bearing autoantibodies that target EEA1 develop neurological disease, we studied the subcellular distribution of EEA1 in neurons and the effect on neurotransmission of purified immunoglobulins from the serum of a patient bearing EEA1 autoantibodies. EEA1 was localized in the soma and in the postsynaptic nerve terminals. Electrophysiological recordings in hippocampal slices including purified EEA1 antibodies in the patch pipette solution, revealed a run-up of AMPA, *N*-methyl-D-aspartate and kainate receptor-mediated excitatory post-synaptic currents recorded from CA3 pyramidal neurons, which was absent in the recordings obtained in the presence of control human immunoglobulin G. Inclusion of human EEA1 antibodies had no effect on inhibitory post-synaptic responses. Recordings in the presence of a dominant-negative C-terminal EEA1 deletion mutant produced a similar effect as observed with human anti-EEA1 antibodies. This specific effect on the excitatory synaptic transmission may be due to the impairment of internalization of specific glutamate receptors and their subsequent accumulation in the synapse. These results may account for the neurological deficits observed in some patients developing EEA1 autoantibodies.

Key words: human autoantibodies, receptor endocytosis, early endosomes, AMPA, NMDA, kainate.

Early endosome antigen 1 (EEA1) was initially identified as an autoantigen in a patient with subacute cutaneous systemic lupus erythematosus (Mu et al., 1995). However, EEA1 is also targeted by the autoantibodies in the sera of the patients with various neurological diseases (Selak et al., 1999). The association of anti-EEA1 antibodies with neurological disease has been inferred not only from clinical studies, but also from the induction of behavioral deficits in mice immunized with EEA1 antigen (Selak and Fritzler, 2004). However, no conclusive evidence for the EEA1 antibody-mediated effects on functional activity of nerve cells has been determined so far.

EEA1 is a 162-kDa hydrophilic peripheral membrane protein present in the cytosol and the cytoplasmic face of early endosomes (Mu et al., 1995; Stenmark et al., 1996). While the role of EEA1 in endocytosis of cultured non-neuronal cell lines has been extensively studied, much less is known about the function of this protein in nerve cells. EEA1 antibodies have been used mostly as research reagents to label early endosomes and to follow the endocytic routes (Ehlers, 2000; Washbourne et al., 2004; Mace et al., 2005). Endocytosis plays important roles in neurotransmission, allowing rapid removal of neurotransmitter receptors (Lin et al., 2000; Ehlers, 2000; Man et al., 2000; Kittler et al., 2000; Sheng and Kim, 2002). EEA1 is a crucial factor controlling vesicle fusion during endocytosis (Patki et al., 1997; Simonsen et al., 1998; Mills et al., 1998; McBride et al., 1999; Rubino et al., 2000) and is a primary antigen targeted by the patients' autoantibodies (Waite et al., 1998; Selak et al., 1999). To get insights into the significance of EEA1 autoantibodies on neurotransmission and in neuropathology, we set out to determine the effects of these human autoantibodies on EEA1 function in neurons.

AMPA receptors (AMPARs) mediate the majority of fast excitatory synaptic neurotransmission in central neurons (Song and Huganir, 2002). Kainate receptors (KARs) also contribute to the post-synaptic responses and can modulate pre-synaptic neurotransmitter release (see Lerma, 2003 for a review). *N*-methyl-D-aspartate receptors (NMDARs) are crucial for the induction of synaptic plasticity and related phenomena (Malenka, 2003). The three types of receptors are subjected to regulated trafficking to EEA1-labeled endosomes, which occurs within minutes and depends on their subunit composition (e.g. Collingridge et al., 2004).

In this study, we found that EEA1 autoantibodies isolated from the serum of a patient with a neurological disease specifically interfere with excitatory neurotransmission as revealed by a run-up of the excitatory post-synaptic current (EPSC) and are internalized by the neurons during a prolonged incubation. A similar stimulatory effect on AMPAR-mediated synaptic currents was observed in the presence of a dominant negative EEA1 mutant, confirming the specificity of the antibody action. Our data indicate that

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Abbreviations: AMPAR, AMPA receptor; DMEM, Dulbecco's Minimal Essential Medium; EEA1, early endosome antigen 1; EPSC, excitatory post-synaptic current; IB, immunoblotting; ICC, immunocytochemistry; IgG, immunoglobulin G; IPSC, inhibitory post-synaptic current; KAR, kainate receptor; NMDAR, *N*-methyl-D-aspartate receptor; PBS, phosphate-buffered saline; PICK1, protein interacting with C-kinase 1; PPF, pair-pulse facilitation; PSD-95, postsynaptic density-95; TBS, Tris buffered saline.

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doi:10.1016/j.neuroscience.2006.10.014

as a part of its general role in endocytosis at a docking/tethering stage of vesicle fusion, EEA1 is actively involved in the modulation of glutamergic neurotransmission. This may elucidate a possible pathogenic role for EEA1 autoantibodies and account for the neurological alterations observed in certain patients bearing these antibodies.

EXPERIMENTAL PROCEDURES

Antibodies

Human sera were obtained and stored as previously described (Selak et al., 1999, 2003). Control sera were randomly selected from a bank of 2000 blood donors or pooled from healthy volunteers. Control normal human immunoglobulin G (IgG) was purchased from Pierce (Rockford, IL, USA). For electrophysiological experiments, antibodies were purified on a protein G sepharose column (Sigma, Madrid, Spain). The bound antibodies were eluted in 100 mM glycine buffer pH 2.5, pH immediately adjusted to 7.4 with 1.5 M Tris buffer pH 9.0 and dialyzed against phosphate-buffered saline (PBS) on the D-Salt Excellulose GF-5 Desalting Column (Pierce). Antibody concentration was measured by the BioRad (Madrid, Spain) protein assay using IgG as a standard and the specificity of the purified antibodies was examined by confocal microscopy, immunoprecipitation (IP) and immunoblotting (IB). For electrophysiological studies and antibody internalization assays, purified human or control IgGs were used at the concentration of $\leq$0.01 mg/ml. For IB and immunolabeling studies human serum was diluted 1/5000. Additional antibodies used were monoclonal anti-EEA1 antibody generated as described previously (Selak et al., 1999; Selak and Fritzler, 2004) (AbCam, Cambridge, UK) (dilution 1/200), affinity-purified goat anti-EEA1 (C-19) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) (dilution 1/400), anti-synaptophysin monoclonal antibody (Synaptic Systems, Gottingen, Germany) (dilution of 1/200 for immunohistochemistry, 1/500 for immunocytochemistry (ICC) and 1/3000 for IB). Monoclonal antibody to postsynaptic density-95 (PSD-95) (Chemicon, Hampshire, UK) was diluted 1/500 for ICC and 1/1000 for IB; rabbit anti-calnexin antibody, and goat anti-protein interacting with C-kinase 1 (PICK1) antibody (Santa Cruz) were used at 1/1000 and 1/400 dilutions, respectively.

Cell lines and hippocampal neurons primary cultures

COS-7 cells were grown in Dulbecco's Minimal Essential Medium (DMEM) with Glutamax (Gibco, Barcelona, Spain), 10% fetal bovine serum, 100 μg/ml streptomycin and 100 μg/ml penicillin at 37 °C in 5% CO_2. For the cell lysate preparation, cells were grown to confluence, washed once in PBS and collected on ice in 1 ml of cold cell lysis buffer (20 mM Hepes pH 7.4, 150 mM KCl, 1 mM EGTA, 1 mM EDTA, 1% Triton X-100). The cell lysate was incubated at 4 °C for 30 min to solubilize cell membranes and then sheared and further solubilized by successive passage through 18 and 26 gauge needles. After centrifugation (10 min at 4 °C at 14,000 r.p.m. in the microcentrifuge), the supernatant was collected and the protein concentration measured by a standard Bradford assay (BioRad).

Hippocampal neurons were prepared from P0 C57 mice. The procedures for handling and killing animals used in this study followed the European Commission guidelines (86/609/CEE). The minimum number of animals needed for significant results was used, and every effort was made to minimize animal suffering. Hippocampi were dissected in ice-cold Hepes-buffered salt solution (HBSS) medium and digested with DNase (5 μg/ml) and trypsin (0.125%) for 30 min at 37 °C. Tissue was dissociated with a flame-polished Pasteur pipette. After centrifugation (300$\times g$ for 5 min), cells were plated on glass coverslips previously treated with poly-D-lysine (60 μg/ml) and laminin (10 μg/ml) and cultured in DMEM supplemented with 10% fetal calf serum, penicillin (100 μg/ml), and streptomycin (100 μg/ml). The medium was replaced after three hours by Neurobasal/B27 medium supplemented with glutamine (2 mM), penicillin (100 μg/ml) and streptomycin (100 μg/ml). Cells were fed by replacing 1/3 of the medium once a week and used for experiments between 14 and 18 DIV.

Immunofluorescence and co-localization

Co-localization of anti-EEA1 with synaptophysin antibodies was performed on hippocampal sections and cultured hippocampal neurons. Hippocampal sections were obtained from adult C57 mice transcardially perfused with 1% heparin followed by perfusion with 4% paraformaldehyde pH 7.4. Brains were immersed in 3% agarose to obtain regular blocks that were further sliced at a thickness of 40 μm using a vibratome. Free-floating slices were washed in PBS and permeabilized by incubation in PBS containing 0.5% Triton X-100 for 30 min. Slices were then incubated 4 h at 4 °C in the blocking buffer (5% normal goat serum, 5% bovine serum albumin and 0.1% Triton X-100 in PBS). Human anti-EEA1 and mouse anti-synaptophysin antibodies were diluted in the blocking buffer and the incubation continued overnight at 4 °C. To detect the bound primary antibodies, slices were incubated for two hours at room temperature with the Alexa 555-conjugated goat anti-human IgG and Alexa 488-conjugated goat anti-mouse IgG (Molecular Probes/Invitrogen, Barcelona, Spain) diluted in the blocking buffer. Following each incubation step, slices were washed 3$\times$15 min in PBS. After the last wash, slices were mounted onto slides in DAPI-supplemented mounting medium (Vectashield: Vector Laboratories, Burlingame, CA, USA) and viewed using a Leica Laser Confocal Microscope. For ICC, COS-7 cells and hippocampal neurons were fixed on the glass coverslips in 4% paraformaldehyde, 10% sucrose at room temperature for 5 min and then permeabilized in 0.2% Triton X-100, PBS for 2 min. Immunolabeling was performed essentially in the same way, except for omitting detergent in the subsequent steps.

Biocytin labeling

To detect the antibodies introduced during electrophysiological recordings, biocytin was dissolved in the recording pipette solution at 1 mg/ml and human anti-EEA1 or normal human IgGs were added at 0.01 mg/ml. Cells were recorded for an average of 30 min, and the recorded slices (see below) were immediately fixed in 4% paraformaldehyde, 10% sucrose for 12 h at 4 °C. Slices were processed for immuno-histochemistry as described above. Biocytin was detected with Alexa 488-conjugated streptavidin (Molecular Probes) and human antibodies with Alexa 555 goat anti-human IgG (Molecular Probes) diluted in the blocking buffer.

Preparation of mouse brain homogenate

Brain tissue isolated from P21 C57 mice was hand homogenized with a Teflon-coated homogenizer in buffer containing 0.32 M sucrose, 10 mM Hepes KOH (pH 7.0), 1 mM EGTA, 0.1 mM EDTA and protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). The homogenate was centrifuged (10 min at 500$\times g$) and the post-nuclear supernatant collected and centrifuged again (40 min at 48,000$\times g$ at 4 °C). The pellet was resuspended in 20 mM MOPS (pH 7.0), 150 mM KCl, 1% Triton X-100 containing protease inhibitor cocktail. Following solubilization, large membrane fractions were removed by centrifugation at 500$\times g$ for 10 min and the protein concentration was determined by BioRad Bradford colorimetric protein assay with bovine serum albumin as a standard.