ELSEVIER

Journal of Neuroscience Methods 160 (2007) 302–308

JOURNAL OF NEUROSCIENCE METHODS

www.elsevier.com/locate/jneumeth

A novel method for monitoring the cell surface expression of heteromeric protein complexes in dispersed neurons and acute hippocampal slices

David Holman *, Jeremy M. Henley

MRC Centre for Synaptic Plasticity, Department of Anatomy, School of Medical Sciences, University of Bristol, Bristol BS8 1TD, UK

Received 16 July 2006; received in revised form 25 September 2006; accepted 26 September 2006

Abstract

The subunit composition of multimeric protein complexes is critical in determining their trafficking and functional properties. Despite there being multiple techniques to investigate the trafficking events of individual subunits there are currently limited means to monitor the trafficking properties of heteromeric protein complexes. Here, we combine surface biotinylation with co-immunoprecipitation to monitor the cell surface expression of native, heteromeric AMPA receptor complexes. Using this method, we demonstrate that the surface levels of GluR1/2 and GluR2/3 complexes are reduced following NMDA-evoked long-term depression (NMDA-LTD) in acute hippocampal slices. Finally, we discuss how this method can be adapted to monitor the cell surface expression of other heteromeric protein complexes.

Keywords: AMPA receptor; Heteromeric; Trafficking; Biotinylation; LTD

1. Introduction

The subunit composition of ionotropic receptors dictates their functional and trafficking characteristics (Palmer et al., 2005). α-Amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptors (AMPARs) are tetrameric, ionotropic glutamate receptors which assemble as a symmetrical dimer of dimers (Mansour et al., 2001). There are four AMPAR subunits (GluR1-4) which display differential expression in different brain regions and exist in multiple splice and edited isoforms (Dingledine et al., 1999). AMPARs are extensively studied in the hippocampus and cerebellum due to their central role in synaptic plasticity (Cull-Candy et al., 2006). In the CA1/CA2 region of rat hippocampus, the majority of AMPARs comprise GluR2 (Ozawa and Iino, 1993) with either GluR1 (GluR1/2) or GluR3 (GluR2/3) (Wenthold et al., 1996). By contrast, at cerebellar stellate cell synapses AMPARs are predominantly GluR2-lacking (Liu and Cull-Candy, 2000).

The subunit composition of AMPARs dramatically affects their function. GluR2 containing AMPARs have low Ca^{2+} permeability so consequently have outwardly rectifying I–V relations, whereas GluR2-lacking AMPARs have high Ca^{2+} permeability and are inwardly rectifying (Burnashev et al., 1992; Hollmann et al., 1991). Subunit composition also appears to affect AMPAR trafficking properties. GluR2-lacking receptors are driven into synapses during the early stages of hippocampal LTP, whereas GluR2-containing receptors replace these after 25 min (Plant et al., 2006). Differential movement of GluR2-lacking and GluR2-containing AMPARs has also been reported following ischemic insult. GluR2-lacking AMPARs are directed towards synapses following oxygen-glucose deprivation, whereas GluR2-containing AMPARs are removed from synaptic sites under the same conditions (Liu et al., 2006).

Recently, we have demonstrated that the surface expression of GluR2 and GluR3, but not GluR1, is reduced following NMDA-LTD induction in acute hippocampal slices. From these data we hypothesised that GluR1/2 and GluR2/3 containing AMPARs are differentially trafficked during LTD (Holman et al., 2006). To test this hypothesis further a method was required to monitor changes in the surface levels of heteromeric AMPAR complexes. In this report we develop a method that combines surface biotinylation with co-immunoprecipitation (co-IP) to assess the surface expression of heteromeric AMPARs in dispersed neurons and acute hippocampal slices. Using this method we determine the surface expression of GluR1/2 complexes under basal conditions and detect changes in the surface levels of GluR1/2 and GluR2/3 complexes following NMDA-LTD induction.

* Corresponding author. Tel.: +44 117 954 6449; fax: +44 117 929 1687.
E-mail address: David.Holman@bris.ac.uk (D. Holman).

0165-0270/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.jneumeth.2006.09.018

2. Materials and methods

2.1. Dispersed neuronal culture and drug treatment

High-density cerebro-cortical cultures were prepared from embryonic day 18 Sprague–Dawley rats. Cortex was dissected in cold Hank's buffered salt solution (Gibco) and then dissociated using trypsin for 10 min at 37 °C. Neurons were then plated onto poly-L-lysine-coated 6 cm dishes (1×10^6 per 6 cm dish) and maintained at 37 °C, 5% CO_2 as described previously (Terashima et al., 2004). After 14 days *in vitro* (DIV), neurons were treated with 25 μM NMDA for 3 min or left untreated. Cultures were then incubated with normal culture medium for 10 min before being immediately placed on ice to prevent further receptor trafficking.

2.2. Biotinylation and lysis of cultures

Neurons were washed twice with ice-cold PBS and incubated with Sulfo-NHS–SS–biotin (Pierce; 0.25 mg/ml in PBS) for 15 min on ice. Neurons were then washed twice with 50 mM NH_4Cl and twice with PBS before being scraped into ice-cold lysis buffer (150 mM NaCl, 20 mM *N*-2-hydroethylpiperazin-*N*′-2-ethanesulfonic acid (HEPES), 1% Triton-X-100, 0.1% SDS, 2 mM EDTA, pH 7.4) containing protease inhibitors (protease inhibitor cocktail with EDTA, 1 in 50; Roche). Samples were then sonicated and placed on a head-over-head shaker for 2 h. Samples were then centrifuged at 100,000 × *g* for 40 min and the pellets were discarded. The protein concentration of the resulting supernatant was determined using a BCA kit (Pierce).

2.3. Acute hippocampal slice preparation

Hippocampal slices (400 μm) were prepared from P21 to P23 male Wistar rats and immediately placed in ice-cold artificial cerebrospinal fluid (ACSF; composition in mM: 124NaCl; 3KCl; 26$NaHCO_3$; 1.25NaH_2PO_4; 2$CaCl_2$; 1$MgSO_4$; 10D-glucose; saturated with 95% O_2 and 5% CO_2). After removing the CA3 region, slices were transferred to a submersion storage chamber where they were maintained in ACSF for 1 h at room temperature.

2.4. Slice treatment for biochemical analysis

To compare multiple NMDA- and ACSF (control)-treated slices simultaneously we used a two-chamber perfusion system. Before treating the slices the cortex was removed leaving the CA1/CA2 region. Alternate slices were then placed into one of two groups, those that would be treated with NMDA and those that would be treated with ACSF. These slices (four to seven slices per group) were then equilibrated at room temperature for 1 h before being placed into the chambers of the two-chamber perfusion system (maintained at 28 °C). Following ACSF perfusion for 40 min, one chamber was perfused with ACSF plus 20 μM NMDA for 5 min, while the other chamber was perfused with ACSF alone. Both sets of slices were then perfused with ACSF for a further 90 min prior to biochemical analysis.

2.5. Slice biotinylation, homogenisation and lysis

Previously it has been shown that acute hippocampal slices can be effectively biotinylated using Sulfo-NHS–SS–biotin. Moreover, it has been demonstrated that biotin can reach all layers of a 400 μm thick slice without labelling intracellular proteins (Holman et al., 2006; Thomas-Crusells et al., 2003). Given these details we were confident that the surface co-IP assay would be suitable for monitoring the surface expression of heteromeric protein complexes in acute hippocampal slices.

Slices were washed once with ice-cold ACSF (5 min) and then incubated with Sulfo-NHS–SS–biotin (Pierce; 0.5 mg/ml in ACSF) for 30 min on ice. Excess biotin was removed by two brief washes with 50 mM NH_4Cl (in ACSF) and two ACSF washes. Slices were then homogenised in 1 ml of homogenisation buffer (320 mM Sucrose; 10 mM Tris; pH 7.4) and centrifuged at 1000 × *g* for 5 min to remove nuclear material and cell debris. Post-nuclear supernatants were centrifuged at 100,000 × *g* for 1 h and supernatants were discarded. The hippocampal cell membranes were resuspended in 1 ml of lysis buffer, sonicated and placed on a head-over-head shaker for 2 h. Samples were then centrifuged at 100,000 × *g* for 40 min and the pellets were discarded. The protein concentration of the resulting supernatant was then determined as described above.

2.6. Streptavidin pull down

Streptavidin beads (40 μl; Sigma) were washed three times with lysis buffer. Lysed biotinylated samples were added to the beads (50 μg total protein), volumes were made up to 200 μl with lysis buffer and mixed on a head-over-head shaker for 4 h. Beads were then centrifuged at 800 × *g*. Loading buffer (4×; containing β-mercaptoethanol) was added to the supernatant to make a final volume of 266 μl. The beads were washed three times with lysis buffer, residual lysis buffer was removed and 133 μl of 2× loading buffer was added. Samples were then heated at 90 °C for 5 min and beads were centrifuged at 800 × *g*. The bead supernatant was then diluted with 133 μl of lysis buffer and stored at −20 °C until further use.

2.7. Immunoprecipitation

Protein G beads (20 μl; Sigma) were washed three times with lysis buffer and centrifuged at 800 × *g*. Lysis buffer (1 ml) containing 2 μg of polyclonal anti-GluR2 (Chemicon) or polyclonal anti-Myc (Upstate) was added to the beads and incubated for 2 h on a head-over-head shaker. Excess antibody was removed by two lysis buffer washing steps. Lysed sample (50–200 μg) was added to the beads and incubated for 6 hr on a head-over-head shaker. Beads were centrifuged at 800 × *g* and the supernatant was used for a second round of GluR2 immunoprecipitation. This additional step was performed to maximise the IP efficiency. After the second round, beads were centrifuged and loading buffer (containing β-mercaptoethanol) was added to the beads and supernatant as described for the streptavidin pull down.