Bidirectional Regulation of Kainate Receptor Surface Expression in Hippocampal Neurons*

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Kainate receptors (KARs) are crucial for the regulation of both excitatory and inhibitory neurotransmission, but little is known regarding the mechanisms controlling KAR surface expression. We used super ecliptic pHluorin (SEP)-tagged KAR subunit GluR6a to investigate real-time changes in KAR surface expression in hippocampal neurons. Sindbis virus-expressed SEP-GluR6 subunits efficiently co-assembled with native KAR subunits to form heteromeric receptors. Diffuse surface-expressed SEP-GluR6a to investigate real-time changes in KAR surface expression. We used super ecliptic pHluorin (SEP)-tagged KAR known regarding the mechanisms controlling KAR surface expression in hippocampal neurons. Sindbis virus-expressed SEP-GluR6 subunits efficiently co-assembled with native KAR subunits to form heteromeric receptors. Diffuse surface-expressed SEP-GluR6 is rapidly internalized following either N-methyl-d-aspartate or kainate application. Sustained kainate or transient N-methyl-d-aspartate application resulted in a slow decrease of baseline surface KAR levels. Surprisingly, however, following the initial loss of surface receptors, a short kainate application caused a long lasting increase in surface-expressed KARs to levels significantly greater than those prior to the agonist challenge. These data suggest that after initial endocytosis, transient agonist activation evokes increased KAR expression and reveal that KAR surface expression is bidirectionally regulated. This process may provide a mechanism for hippocampal neurons to differentially adapt their physiological responses to changes in synaptic activation and extrasynaptic glutamate concentration.

Kainate receptors (KARs) are intimately involved in the regulation of both excitatory and inhibitory neurotransmission. The rates, extent, and location of KAR trafficking and plasma membrane expression are modulated by interactions with intracellular proteins, which orchestrate KAR function and plasticity (1–4). In hippocampal neurons, internalized GluR6-containing KARs are sorted into recycling or degradative pathways depending on the endocytic stimulus. Sustained kainate activation causes a protein kinase A-independent but protein kinase C-dependent internalization of KARs targeted to lysosomes for degradation. In contrast, N-methyl-d-aspartate receptor (NMDAR) activation evokes protein kinase A- and protein kinase C-dependent endocytosis of KARs to early endosomes with subsequent reinsertion in the plasma membrane (5). Posttranslational protein modification by SUMO (small ubiquitin-like modifier) is also involved in KAR endocytosis (6). GluR6 SUMOylation in hippocampal neurons occurs at the plasma membrane in response to direct activation of the receptors, leading to their rapid removal from the cell surface (7). In dorsal root ganglion neurons, GluR5-containing KARs are also internalized via a protein kinase C-dependent mechanism to regulate the number of surface-expressed KARs (8), and protein kinase C activity participates in KAR trafficking in the perirhinal cortex, where it is involved in NMDAR-independent KAR-mediated long term depression (9).

The aim of this study was to define the properties of KAR trafficking and surface expression in dendrites under basal and stimulated conditions using super ecliptic pHluorin (SEP)-tagged GluR6 subunit. We show that SEP-GluR6 forms heteromeric assemblies with native KAR subunits and that the plasma membrane KAR population is differentially regulated by NMDA or kainate stimulation. Both sustained KAR and transient NMDAR stimulations lead to decreased surface GluR6-containing KARs, whereas transient kainate application results in a long lasting increase in surface levels of KARs, indicating that KAR surface expression is bidirectionally regulated.

EXPERIMENTAL PROCEDURES

Dissociated Hippocampal Cultures—Primary hippocampal neuron cultures are a well-established and extensively used model for studying receptor trafficking that allows access to the plasma membrane for antibody labeling, biotinylation, and surface fluorophore imaging. Hippocampal cultures were prepared as described previously (5). Cells were plated at a density of 500,000/35-mm dish or 50,000 onto 22-mm coverslips coated with poly-L-lysine (Sigma).

Plasmid and Sindbis Virus Preparation—SEP-Myc-GluR6a was constructed by inserting the SEP fragment at the 5'-ClaI site of the Myc tag of pcDNA3-Myc-GluR6. To remove unnecessary 3'-untranslated region fragment, a new NotI site was introduced after the stop codon of GluR6 in pcDNA3-Myc-GluR6 by site-directed mutagenesis using the QuickChangeXL kit (Stratagene). The D718I-NotI insert fragment containing the entire SEP-GluR6a cDNA was then subcloned into the plasmid and Sindbis Virus Preparation. The on-line version of this article (available at http://www.jbc.org) contains a supplemental legend and Movies 1 and 2. 
cloned into the same sites of the entry plasmid pENTRIA (Invitrogen). The Sindbis expression vector was generated by site-specific recombination between the entry plasmid and pSinRep5-DEST by using LR Clonase (Invitrogen) according to the manufacturer’s protocol. Attenuated Sindbis virus expressing SEP-GluR6a was prepared and used as described previously (6). Neurons were transduced at a multiplicity of infection of 1 at 18–20 DIV and then returned to the incubator for an additional 20–30 h before use.

Biotinylation Experiments—Live hippocampal neurons (18–21 DIV) were preincubated in Neurobasal medium in the presence of TTX (2 μM) for 10 min at 37 °C. Neurons were then washed in Earle’s-TTX buffer (25 mM HEPES, Tris-buffered to pH 7.4, 140 mM NaCl, 5 mM KCl, 1.8 mM CaCl₂, 0.8 mM MgCl₂, 5 mM glucose, and 2 μM TTX) and subsequently incubated in Earle’s-TTX buffer at 37 °C in the absence or presence of drugs for the times indicated under “Results.” After three washes in ice-cold phosphate-buffered saline (PBS), plasma membrane proteins were biotinylated using the membrane-impermeant sulfo-NHS-SS-biotin (0.15 mg/ml; Pierce) in PBS for 10 min on ice. Labeled neurons were then washed with cold Tris-buffered saline (25 mM Tris, pH 7.4, 137 mM NaCl, 5 mM KCl, 2.3 mM CaCl₂, 0.5 mM MgCl₂, and 0.143 g/liter Na₂HPO₄) to quench free biotin-reactive groups. After two further washes with PBS on ice, cells were lysed in extraction buffer (10 mM Tris-HCl, pH 7.5, 10 mM EDTA, 1% Triton X-100, 0.1% SDS, and 1% mammalian protease inhibitor mixture (Sigma)). Following centrifugation (15,000 × g, 20 min, 4 °C), supernatants containing equal amounts of protein were incubated with streptavidin beads to immunoprecipitate the surface-biotinylated proteins. Following extensive washes in extraction buffer, proteins were eluted by boiling in reducing sample buffer, resolved by SDS-PAGE, and immunoblotted using anti-GluR6/7 antibody (1:2000; Upstate Biotechnology). Standard actin controls were included to ensure that there was no biotinylation of intracellular proteins. In no case was actin retained on streptavidin.
pulldowns (see Fig. 1B), confirming that there was no unsppecific uptake of biotin. Band intensities were quantified using NIH ImageJ 1.38 software and normalized to the total receptor band.

Exocytosis Assays—Live TTX-treated hippocampal neurons (18–21 DIV) were rinsed three times with ice-cold PBS and incubated with ice with 1.5 mg/ml sulfo-NHS-acetate (Pierce) in PBS at 4 °C for 20 min. Neurons were washed twice with PBS and twice with Tris-buffered saline to quench remaining free reactive groups. Cells were then stimulated in Earle’s-TTX and twice with Tris-buffered saline to quench remaining free groups. Cells were then stimulated in Earle’s-TTX and twice with Tris-buffered saline to quench remaining free

Fluorescence Imaging of SEP-GluR6 in Fixed Neurons—Live SEP-GluR6-expressing neurons were surface-labeled at room temperature for 20 min with both a chicken anti-GluR6 N-terminal antibody (1:4000) (5, 6, 10) and an Alexa Fluor 647-coupled anti-GFP antibody (1:1000; Molecular Probes). After extensive washes in PBS at room temperature, cells were fixed with 4% paraformaldehyde in PBS for 15 min. Cells were then mounted in Mowiol (Sigma), and images were taken with an inverted confocal microscope (Zeiss Axiocvert 200M) as previously described (5, 6).

Statistical Analysis—Statistical analyses were calculated using Prism 4 (GraphPad Software, Inc.). Data are expressed as means ± S.E. Unpaired Student’s t tests and one-way analysis of variance were performed with a Newman–Keuls post-test for multiple comparison data sets when required.

RESULTS

Surface GluR6 Expression Is Differentially Regulated—KARs are activity-dependently internalized in hippocampal neurons (5). To investigate the effects of NMDA and kainate receptor activation on the surface-expressed KARs, we designed a surface protein biotinylation-based assay (Fig. 1A). Both NMDA and kainate application invoke KAR endocytosis (5, 6), but the precise mechanisms are not well defined. As shown in Fig. 1 (B and C), both sustained KAR and transient NMDAR activation induced an ~40% reduction of native GluR6 subunits at the cell surface. In contrast, transient (3 min) direct KAR activation by kainate was followed by a time-dependent increase in GluR6-containing KAR at the plasma membrane (125.2 ± 4.01% 20 min after transient kainate treatment) with no alteration in the total level of GluR6 (Fig. 1, D and E). These results show that unlike NMDAR and sustained KAR stimulation, which cause down-regulation of KAR surface content, direct transient KAR activation up-regulates plasma membrane GluR6-containing KARs.

Live Cell Confocal Imaging—Protopocols were as previously described (11, 12). Briefly, live SEP-GluR6-expressing neurons (18–21 DIV) were preincubated in Neurobasal medium containing TTX (2 μM) for 10 min and then transferred in Earle’s-TTX buffer for live confocal imaging experiments. Neurons were placed on the heated stage (set at 37 °C) of an inverted Zeiss Axiovert microscope and were continuously perfused at 2 ml/min with warm Earle’s-TTX solution. For a low pH external solution, equimolar MES was used instead of HEPES, and pH was adjusted to 6.0. NH4Cl (50 mM) was used in place of equimolar NaCl to collapse the pH gradient. Fluorescence was excited using a 63× water-immersion objective (numerical aperture = 1.2) by 488 nm laser light, and emission was detected through a 505-nm long pass filter. Time series were collected as repetitively scanned image stacks. Image stacks were then flattened using the maximum projection algorithm from the Zeiss LSM software. All SEP-GluR6 experiments included a brief (10 s) low pH wash at the beginning to ensure that the fluorescence from the area of interest came from surface-expressed KARs.
SEP-GluR6 Is Trafficked to the Plasma Membrane and Assembles with Native KAR Subunits—To further investigate the mechanisms by which transient KAR activation induces increased KAR surface expression, we tagged the KAR subunit GluR6a with SEP (Fig. 2A), an enhanced version of pH-sensitive GFP (13). When expressed in COS-7 cells, fluorescent SEP-GluR6 forms fully functional homomeric receptors with electrophysiological properties indistinguishable from wild-type untagged GluR6a (data not shown). The validated SEP-GluR6 was cloned into Sindbis virus for efficient neuronal expression (6, 11, 12). Using Sindbis virus at a multiplicity of infection of 1, ~90% of 19–21 DIV-cultured hippocampal neurons expressed the SEP-GluR6 transgene 20–28 h after infection. Importantly, the total levels of recombinant SEP-GluR6 protein were similar to the levels of native GluR6 subunits at 24 h after transduction (Fig. 2, B and C). Furthermore, the levels of surface-expressed SEP-GluR6 and endogenous GluR6 were also similar, accounting for ~25% of the total SEP-GluR6 or GluR6, respectively, indicating that the SEP tag does not affect GluR6 forward trafficking (Fig. 2B).

We have shown previously that virally expressed AMPAR subunits do not efficiently assemble with native subunits (14). To determine whether this is the case for KARs, we performed co-immunoprecipitation assays on SEP-GluR6-expressing hippocampal neurons using anti-GFP, anti-GluR6, anti-KA2, and anti-GluR1 antibodies. In contrast to recombinant AMPAR subunits, our data demonstrate that SEP-GluR6 does heteromultimerize with native GluR6 and KA2 KAR subunits but not with AMPAR subunit GluR1 in hippocampal neurons (Fig. 2, C and D).

As expected, SEP-GluR6 fluorescence co-localized with both anti-GluR6 N-terminal (5, 6, 10) and anti-GFP antibodies in fixed non-permeabilized neurons (Fig. 2E). We also investigated the co-localization of SEP-GluR6 with synaptic markers SV2A and PSD95 (Fig. 2, F and G). Entirely consistent with a previous report on native GluR6 in hippocampal neurons (15), there was a distinct but limited co-localization of SEP-GluR6 with 20.60 ± 4.30% and 22.74 ± 5.04% for SV2A and PSD95 markers, respectively.

SEP-GluR6 Reports Fluorescent Surface-expressed KARs—We next investigated the surface expression of Sindbis virus-expressed SEP-GluR6 in living hippocampal neurons. As shown in Fig. 3 (A and B), the fluorescence can be almost entirely attributed to surface-expressed SEP-GluR6 because
Real-time Imaging of KAR Surface Trafficking

transient exposure to external pH 6.0 buffer rapidly eclipsed the fluorescent signal. To reveal total levels of SEP-GluR6 fluorescence (surface plus intracellular), cells were transiently exposed to 50 mM NH4Cl-containing buffer to equilibrate all internal pools to pH 7.4. Application of pH 6.0 buffer in the presence of 50 mM NH4Cl eclipsed all SEP fluorescence (Fig. 3, A and B). Additional confirmation that SEP-GluR6 was surface expressed was obtained by surface labeling SEP-GluR6 with Alexa Fluor 647-conjugated anti-GFP antibody (red) in a dendrite of a living neuron. Rapid exchange of pH 7.4 to pH 6.0 resulted in the reversible loss of the intrinsic SEP fluorescence with no changes in antibody staining (Fig. 3C). Both bright punctate areas of fluorescence and diffuse lower levels of fluorescence (Fig. 3D) were observed. The punctate fluorescence was directly facing presynaptic FM4-64-labeled active terminals (Fig. 3E), consistent with postsynaptic surface KARs. These results demonstrate that SEP-GluR6 is correctly targeted to synaptic compartments.

Effects of NMDA and Kainate on Diffuse and Punctate Surface SEP-GluR6 Fluorescence—We next determined the properties of NMDA- and kainate-evoked KAR endocytosis (Fig. 4, A–D). The levels of surface SEP-GluR6 were monitored at defined puncta on spines and at diffuse non-spiny dendritic regions. Dramatically different profiles were observed for these two pools of receptors. Diffuse SEP-GluR6 fluorescence was rapidly decreased by NMDA application (Fig. 4, A and C; supplemental Movie 1) with recovery starting soon after NMDA washout and return to base-line levels within 10 min. Punctate SEP-GluR6 fluorescence was also reduced by NMDAR activation but with a much slower onset and a less marked decrease. Furthermore, the punctate SEP-GluR6 fluorescence continued to decrease after removal of NMDA, and the gradual reduction persisted throughout the 12-min time course of the experiments (Fig. 4, A and C). None of these NMDA effects occurred when neurons were preincubated with the NMDAR antagonist d(-)-2-amino-5-phosphonopentanoic acid (data not shown).

Kainate application also evoked very different changes in SEP-GluR6 fluorescence (Fig. 4, B and C). Immediately upon exposure to kainate, there was a rapid and substantial decrease in diffuse SEP-GluR6 fluorescence, and levels continued to decrease, albeit more slowly, during the 3-min kainate incubation period. In the continued presence of kainate, the levels of surface SEP-GluR6 remained decreased (data not shown). Following kainate removal, diffuse SEP-GluR6 recovered and then increased to values similar to base-line levels. Intriguingly, despite the initial loss of diffuse fluorescence, kainate application did not decrease punctate spine-associated SEP-GluR6 fluorescence (Fig. 4, B and C; supplemental Movie 2). Rather, there was a gradual increase in punctate fluorescence that started after removal of kainate and was maintained at 12 min. The fact that both the punctate and diffuse fluorescence increased after kainate removal suggests that exocytosis may occur simultaneously at spiny and non-spiny areas of dendrites (Fig. 4C).

To determine whether the increase in surface KAR following short exposure to kainate (Figs. 1D and 4, B and C) was due to KAR exocytosis, we designed a plasma membrane receptor insertion assay using a modified cell surface biotinylation experimental approach (Fig. 4D) (16). Surface-expressed proteins were saturated with membrane-impermeant sulfo-NHS-acetate and then stimulated with kainate for 3 min. Control neurons from the same cultures were incubated with sulfo-NHS-acetate but not exposed to kainate. After treatment, neurons were surface-biotinylated at various time points to label newly exocytosed native KARs. Only de novo exocytosed KARs were labeled because previously surface-expressed/recycled receptors were covalently modified by the acetate group (Fig. 4, D and E). Transient kainate treatment led to a slow and persistent increase in exocytosis of new KARs, whereas no de novo insertion of native KARs was observed in control untreated neurons during the time course of the experiment (Fig. 4E).

**DISCUSSION**

The fine-tuning of functional glutamate receptors is fundamental for the regulation of synaptic strength and cell excitability. Endocytosis, recycling, exocytosis, and lateral diffusion all contribute to changes in the surface expression and compartmentalization of membrane receptors (for a recent review, see Ref. 17). Here we have used SEP-GluR6 to show that surface expression of GluR6-containing KARs is dynamically regulated. Sindbis virus-expressed SEP-GluR6 represents a valuable tool for the study of real-time dynamic movement of KARs in neurons. SEP-GluR6 efficiently assembles in heteromultimers with endogenous KAR subunits and consequently acts as an effective reporter for KAR containing native subunits. Thus, SEP-GluR6 trafficking is subject to regulation by interactions occurring at the GluR6 subunit as well as interactions occurring at other subunits within the multimeric subunit complex. Taken together, our data indicate that analysis of the SEP fluorescent signal provides a faithful readout for the behavior of endogenous GluR6-containing KARs.

NMDAR stimulation elicited a decrease in fluorescence of punctate and diffuse SEP-GluR6 populations, similar to responses observed previously for the AMPAR subunit pHLucorin.
GluR2 (18). Unlike NMDAR and sustained KAR stimulation, which cause down-regulation of surface KARs, transient KAR activation up-regulates plasma membrane GluR6-containing KARs. During a 3-min kainate pulse, there was a rapid and substantial decrease in diffuse SEP-GluR6 fluorescence due to their endocytosis but no change in punctate spine-associated SEP-GluR6 fluorescence. After kainate removal, however, both punctate and diffuse SEP-GluR6 increased with similar time courses. This suggests either that exocytosis occurs simultaneously at spiny and non-spiny areas of dendrite or that, following transient kainate stimulation, KARs that remain on the non-spiny dendritic shaft membrane are recruited to spines and that this lateral diffusion is more than compensated for by exocytosis to the shaft. We do not attribute these differential trafficking events to differences in degradation rates because we previously showed that the time course of KAR degradation is in the hour time scale (5). Possible mechanisms underlying this could include that the kainate-induced current is not desensitized and that the residual current produces the long term effects. Alternatively, the desensitized state of the KAR might, under sustained kainate application, contribute to differences in the trafficking properties. However, we propose that differential modulation of posttranslational GluR6 modification occurs in the continuous presence of the agonist.

KAR activation induces SUMOylation of GluR6 at the plasma membrane of hippocampal neurons, leading to their endocytosis (7). This raises the possibility that after endocytosis under sustained KAR activation, SUMOylated KARs are retained in intracellular compartments and can subsequently be targeted for degradation at lysosomes. Furthermore, de-SUMOylation after a relatively brief agonist application may provide a downstream signal for KAR retrieval in a recycling pool and exocytosis because kainate removal results in increased surface expression of KARs. Indeed, we demonstrated previously that a fraction of KARs are subject to lysosomal degradation (5). Relevant to this, high frequency stimulation with pulses of 50 μM kainate triggers a rundown of kainate-induced GluR5-containing KAR responses in dorsal root ganglion neurons, whereas this rundown does not occur with kainate pulses that were delivered at 5-min intervals (8). Thus, both the type and the frequency of stimuli determine the regulation of KAR surface expression. This is of particular importance with respect to KAR physiopathologies. For example, a prominent feature of ischemia is massive glutamate release that leads to excessive glutamate receptor activation and consequent excitotoxic cell death. It would therefore be advantageous to reduce KAR signaling in such circumstances.

Another important implication of our findings comes from the fact that during cerebral development, filopodial motility is differentially regulated by KARs. In young immature hippocampal slices, synaptic stimulation of KARs enhances filopodial motility, but it inhibits it in mature slices (19). Transient stimulation of GluR6-containing KARs evokes a fast and reversible growth cone stalling (20), a prerequisite for synapse stabilization. Our data show that transient KAR activation leads to increased receptor surface expression. Thus, in young neurons, short bursts of glutamate could lead to raised levels of surface-expressed GluR6-containing KARs, mediating the stalling of growth cones and thereby facilitating the establishment/rupturbation of novel synaptic structure.

In summary, we envisage that the bidirectional regulatory mechanism we describe here controls the levels of plasma membrane-expressed KARs in response to changing environmental conditions. Further investigation will be required to determine which KAR interactors and/or posttranslational modifications are involved in these differential trafficking events.

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