Intracellular trafficking of ionotropic glutamate receptors is controlled by multiple discrete determinants in receptor subunits. Most such determinants have been localized to the cytoplasmic carboxyl-terminal domain, but other domains in the subunit proteins can play roles in modulating receptor surface expression. Here we demonstrate that formation of an intact glutamate binding site also acts as an additional quality-control check for surface expression of homomeric and heteromeric kainate receptors. A key ligand-binding residue in the KA2 subunit, threonine 675, was mutated to either alanine or glutamate, which eliminated affinity for the receptor ligands kainate and glutamate. We found that plasma membrane expression of heteromeric GluR6/KA2(T675A) or GluR6/KA2(T675E) kainate receptors was markedly reduced compared with wild-type GluR6/KA2 receptors in transfected HEK 293 and COS-7 cells and in cultured neurons. Surface expression of homomeric KA2 receptors lacking a retention/retrieval determinant (KA2-R/A) was also reduced upon mutation of Thr-675 and elimination of the ligand binding site. KA2 Thr-675 mutant subunits were able to co-assemble with GluR5 and GluR6 subunits and were degraded at the same rate as wild-type KA2 subunit protein. These results suggest that glutamate binding and associated conformational changes are prerequisites for forward trafficking of intracellular kainate receptors following multimeric assembly.

Modulation of the biosynthesis and intracellular trafficking of ionotropic glutamate receptors underlies plasticity at some excitatory synapses in the mammalian central nervous system (1, 2). Ionotopic glutamate receptors of the AMPA, kainate, and NMDA subtypes are assembled from component subunits into tetrameric receptor complexes in the endoplasmic reticulum. The nascent receptors are then transported forward through the secretory pathway to their sites of functional activity, which in neurons can be in postsynaptic densities or at extrasynaptic sites. 

Received for publication, October 12, 2004, and in revised form, December 1, 2004
Published, JBC Papers in Press, December 6, 2004, DOI 10.1074/jbc.M411549200

Lokanatha Valluru‡, Jian Xu§, Yongling Zhu§, Sheng Yan‡, Anis Contractor‡, and Geoffrey T. Swanson‡∥

From the ‡Department of Pharmacology and Toxicology, University of Texas Medical Branch, Galveston, Texas 77555-1031, the ¶Molecular Neurobiology Laboratory, The Salk Institute, La Jolla, California 92037, and the ¶Department of Physiology, Northwestern University Feinberg School of Medicine, Chicago, Illinois 60611

The abbreviations used are: AMPA, N-methyl-D-aspartate; PBS, phosphate-buffered saline; PDI, protein disulfide isomerase; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione.

Originally thought to be relatively static compared with AMPA receptors (6), but more recent reports have demonstrated dynamic regulation of NMDA receptor trafficking in ways that might affect induction of synaptic plasticity (Refs. 7 and 8; reviewed in Ref. 9). Relatively few examples of activity-dependent changes in neuronal kainate receptors have been reported (10, 11), and the cellular mechanisms that underlie those functional changes remain obscure.

Transit of ionotopic glutamate receptors through the secretory and endocytic pathways is primarily controlled by interactions between cellular chaperone proteins and discrete motifs on the receptor subunits, which are located predominantly on the cytoplasmic carboxyl-terminal domain. These domains are particularly important in the control of receptor egress from the endoplasmic reticulum (ER) after receptor assembly. A number of critical ER-trafficking determinants have been identified in glutamate receptor subunits, which include arginine-rich sequences in NR1, GluR5–2b, GluR5–2c, GluR6, and KA2 subunits that promote ER retention/retrieval (12–16) or export (16, 17), PDZ binding domains in GluR2 AMPA receptor subunits and splice variants of NR1 that control ER export (12, 13, 18), and juxtamembrane determinants that promote export of NR2B (19) and GluR2 (20) but retention of GluR2 subunit-containing receptors (18). In addition to cytoplasmic determinants, glutamate receptors in the ER are subject to a resident quality control system that verifies that the proteins are properly folded and assembled before export to Golgi compartments (21). Non-cytoplasmic determinants in the transmembrane and extracellular domains within the receptor subunits are participants in this quality control process. RNA editing at the Q/R site of GluR2 AMPA receptor subunits reduces calcium permeability (22) and channel conductance (23) and was recently shown to control trafficking and assembly in the ER by preventing tetramerization of all-edited subunits into a functional receptor (18, 24). In addition, it has been proposed that glutamate binding to receptors in the ER acts as a checkpoint for forward trafficking by probing receptor functionality, because...
mutations that eliminate binding in the *Caenorhabditis elegans* GLR-1 AMPA receptor subunit and the GluR6 kainate receptor subunit promoted retention of these receptors (25, 26).

Here we have tested the hypothesis that ligand binding is a critical checkpoint in kainate receptor trafficking by introducing a mutation into the ligand binding domain of the KA2 subunit, a subunit that does not form functional homomeric kainate receptors (14, 27). Assembly of KA2 subunits with GluR5, GluR6, or GluR7 subunits produces functional heteromeric receptors with distinct pharmacological and physiological characteristics (27, 28). KA2 homomers are retained in the ER because this subunit contains an arginine-based retrieval/retention motif in the cytoplasmic domain. Mutation of this trafficking determinant releases homomeric KA2 receptors from the ER, but despite plasma membrane expression the receptors do not gate currents in response to high concentrations of glutamate (14). We found that elimination of the glutamate binding affinity in the KA2 subunit caused retention of this subunit as well as heteromeric kainate receptor complexes that incorporated the mutated subunit in both transfected cell lines and neurons. Retention was not accompanied by an increased degradation rate, suggesting that the mutation did not cause gross misfolding of the receptors. These data support the hypothesis that glutamate binding is a checkpoint in the biosynthesis of kainate receptors. Further, we suggest that binding-related conformational changes in the receptor structure, rather than ion permeation through the channel, are critical for this quality control pathway.

**MATERIALS AND METHODS**

**Molecular Biology**—My-CA2, myc-CA2-R/A, and myc-CA2ΔR5 cDNAs were obtained from John Marshall (Brown University, Providence, RI), and GFP-GluR6 cDNA was obtained from Steve Heinemann (Salk Institute, La Jolla, CA). KA2(T675E) and KA2(T675A) cDNAs were obtained from John Marshall (Brown University, Providence, RI). Extracellular solution contained 150 mM NaCl, 2.8 mM KCl, 1.8 mM CaCl2, 1 mM MgCl2, 10 mM HEPES, and 5 mM EGTA (adjusted to pH 7.3). 3-(N-Morpholino)propanesulfonic acid (MOPS) was used to adjust the pH. Cells were maintained in a humidified chamber at 37 °C, rinsed twice with PBS, and fixed with 4% paraformaldehyde/4% sucrose in PBS for 20 min at room temperature. The coverslips were then blocked in PBS containing 10% bovine serum albumin for 2–3 h at room temperature, mouse anti-myc (1:200) or anti-myc (1:100) antibody for 30 min at 4 °C, washed once with ice-cold PBS, cells were incubated with the appropriate fluorescence-conjugated secondary antibodies (Alexa Fluor 594 or 633, Molecular Probes) for 1 h at room temperature. Surface and intracellular immunofluorescence was captured with a Zeiss LSM510 confocal microscope (Carl Zeiss, Thornwood, NY). For clarity in presentation, the long red fluorescence emitted by Alexa 633 was false-colored as cyan in the figures. Representative images in the figures are single optical slices with thickness of 0.38–0.40 μm.

**Enzyme-linked Immunosorbent Assays**—ELISAs were performed using a described protocol (29). Cells were washed twice with cold PBS, and fixed in 4% paraformaldehyde in PBS for 1 h at room temperature. Cells were then blocked in PBS containing 1% bovine serum albumin and 10% normal donkey serum for 1 h at room temperature. Cells were incubated at 4 °C with anti-GFP antibody (1:200) or anti-myc (1:400) antibody for 1 h, washed twice with cold PBS. Cells were incubated with the appropriate fluorescence-conjugated secondary antibodies (Alexa Fluor 488, Molecular Probes) for 1 h at room temperature. Surface and intracellular immunofluorescence was captured with a Zeiss LSM510 confocal microscope (Carl Zeiss, Thornwood, NY). For clarity in presentation, the long red fluorescence emitted by Alexa 488 was false-colored as cyan in the figures. Representative images in the figures are single optical slices with thickness of 0.38–0.40 μm.

**Electrophysiology**—HEK 293 cells were maintained and transfected as described previously (17). Receptor cDNAs were transfected in combination with a plasmid DNA containing enhanced GFP (typically 0.3 and 0.1 μg, respectively). For experiments with heteromeric receptors, GluR5, GluR6, KA2, and enhanced GFP cDNAs were transfected in a 1:6:1 ratio. Patch clamp recordings were performed 2 days after transfection using a Zeiss Axioskop FS2 microscope (Carl Zeiss), Axopatch 200B amplifier, and pClamp 9 software (Axon Instruments, Foster City, CA). Extracellular solution contained 150 mM NaCl, 2.8 mM KCl, 1.8 mM CaCl2, 1 mM MgCl2, and 5 mM HEPES (adjusted to pH 7.3). Intracellular solution contained 110 mM CsF, 30 mM CsCl, 4 mM NaCl, 0.5 mM CaCl2, 10 mM HEPES, and 5 mM EGTA (adjusted to pH 7.3). Glutamate (10 mM) and (S)-AMPA (300 μM) were applied to transfected cells using a fast application system described previously (17). Analysis was performed off-line using Clampfit software (Axon Instruments).

**Immunoprecipitation and Western Blots**—Transfected COS-7 cells were washed twice with cold PBS and lysed in 0.1 or 0.5 ml of lysis buffer (50 mM Tris, 150 mM NaCl, 0.5% Nonidet P-40, 2 mM 4-(2-aminoethyl)benzenesulfonyl fluoride HCl, 1 mM EDTA, 130 μg bestatin, 14 μM E-64, 1 mM leupeptin, 0.3 μM aprotinin). Supernatants were obtained after lysis and centrifugation at 15,000 rpm at 4 °C for 20 min. Immunoprecipitations were performed by incubating lysate supernatants with anti-GFP antibody (2.5 μg; JL-8, BD Biosciences Clontech, Palo Alto, CA) followed by incubation with 50 μl of 50% protein A/G-Sepharose (Amersham Biosciences) slurry overnight. After four washes in lysis buffer, bound proteins were eluted from the beads by boiling in 2× sample buffer and then separated by electrophoresis on 8% SDS-PAGE gels. Proteins were electro-transferred onto nitrocellulose membranes and probed with anti-myG antibody (0.1 μg/ml, Upstate Biotechnology Inc., Lake Placid, NY) overnight at 4 °C. Immunoreactive bands were visualized using horseradish peroxidase-conjugated donkey anti-rabbit secondary antibody with the enhanced chemiluminescence (ECL) detection technique (Amersham Biosciences).

**Immunolocalization of Receptors**—Cell surface receptors were detected in transfected COS-7 cells following incubation with polyclonal anti-GFP antibody (1:500) or polyclonal anti-myc antibody (1:500) for 1 h at 4 °C. The cells were washed with cold PBS and fixed with 4% paraformaldehyde/4% sucrose in PBS for 20 min at room temperature. After fixation, the cells were washed with PBS and incubated with fluorescence-conjugated goat secondary antibody (Alexa Fluor 594, Molecular Probes, Eugene, OR) overnight at 4 °C. Immunofluorescence was detected using the Zeiss LSM510 confocal microscope (Carl Zeiss, Thornwood, NY). For clarity in presentation, the long red fluorescence emitted by Alexa 594 was false-colored as cyan in the figures. Representative images in the figures are single optical slices with thickness of 0.38–0.40 μm.
bated on ice for 10 min in lysis buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 1% Triton X-100) with a mixture of protease inhibitors (Roche Applied Science) after which solubilized extracts were collected for immunoprecipitation. Proteins were then immunoprecipitated with polyclonal anti-myc antibody, resolved with SDS-PAGE, and quantified on a PhosphorImager 420S (Amersham Biosciences). The measured densities of at each time point were normalized to the image density at time point zero.

Radioligand Binding Assays—Crude membrane fractions were prepared from transiently transfected COS-7 cells by first homogenizing in buffer (50 mM Tris-Cl, pH 7.4, 0.32 M sucrose, pH 7.4), centrifuging at 800 × g for 10 min at 4 °C, and re-centrifuging the supernatant at 13,000 × g for 20 min at 4 °C. The pellet was resuspended in 2 ml of homogenizing buffer, and the process was repeated twice. The final membrane pellet was resuspended in 50 mM Tris-Cl, pH 7.4, and 25 μg of membrane protein was used in radioligand binding assays with 5, 10, and 50 nM [3H]kainate. Nonspecific binding was measured in the presence of 1 mM glutamate. Binding was carried out for 1 h at 4 °C before rapid filtration on Whatman GF/C glass filters through a Brandel Cell Harvester (Biomedical Research and Development Laboratories, Gaithersburg, MD), three washes with cold 0.9% saline solution, addition of scintillation fluid and counting on a Beckman LS5000 TD scintillation counter (Beckman Instruments).

RESULTS

To determine how elimination of glutamate binding affinity of KA2 subunits affected the biosynthesis and pharmacological properties of heteromeric kainate receptors, we first altered a critical threonine residue in the putative binding domain of the KA2 subunit to either an alanine (T675A) or a glutamate (T675E) using site mutagenesis of the receptor cDNA. The hydroxyl moiety of the equivalent threonine in GluR2 (T655) forms an essential hydrogen bond with the γ-carboxyl group of glutamate and kainate (30). Mutation of the equivalent residue in the NR2A subunits increased the EC_{50} for glutamate by three orders of magnitude (31) and eliminated specific binding of [3H]kainate to chick kainate-binding protein (32). Consistent with these results, neither KA2(T675A) nor KA2(T675E) receptors bound [3H]kainate in radioligand assays using membrane preparations from transfected COS-7 cells; that is, total binding was not different from nonspecific binding at any concentration tested (5–50 nM, data not shown). In contrast, specific binding of [3H]kainate was robust in membranes from cells expressing wild-type KA2 subunits and was consistent with the K_{d} of ~15 nM described previously (27). Thus, mutation of Thr-675 eliminated detectable ligand binding to the KA2 subunit.

To determine how the loss of a functional glutamate binding site affected heteromeric kainate receptors, we co-transfected HEK-293 cells with myc-tagged KA2, KA2(T675A), and KA2(T675E) cDNAs with GluR6 cDNAs. Currents arising from heteromeric GluR6/KA2 receptors were distinguished in patch clamp recordings on the basis of their rapid desensitization in response to a 100-ms application of 10 mM glutamate (Fig. 1A), as described previously (33). In addition, we selected only those cells in which peak current amplitudes elicited by 300 μM S-AMPA were at least 25% of those evoked by glutamate to exclude cells expressing predominantly homomeric GluR6 receptors, which can occur even when cDNA ratios are as high as 1:6 (GluR6:KA2). Consistent with earlier reports, GluR6/KA2 receptor currents desensitized with a τ_{des} of 1.6 ± 0.1 ms (n = 3) in cells that exhibited robust responses to (S)-AMPA (Fig. 1, A and B). In contrast, we could not detect AMPA-evoked currents from GluR6/KA2(T675A) and GluR6/KA2(T675E) cells (n = 6, Fig. 1A), suggesting that the mutations also eliminated low affinity binding of AMPA to the KA2 subunit. Glutamate-evoked currents present in a subset of cells expressing the mutant heteromeric receptors desensitized at a rate similar to homomeric GluR6 receptors (GluR6/KA2(T675A): τ_{des} = 5.3 ± 0.6 ms, n = 3; GluR6/KA2(T675E): τ_{des} = 5.6 ± 0.6 ms, n = 4; GluR6: τ_{des} = 4.1 ± 0.2 ms (34)) (Fig. 1B).

We next determined if mutation of the ligand binding site occluded multimeric assembly of receptor subunits, thereby preventing formation of heteromeric receptors. Membranes were prepared from HEK 293 cells co-expressing GFP-tagged GluR6 and myc-tagged KA2 receptor subunits, and receptor proteins were isolated by immunoprecipitation with an anti-myc antibody. Proteins were separated by denaturing SDS-PAGE and transferred to nitrocellulose membranes for Western analysis with an anti-myc primary antibody (Fig. 1C). Myc-KA2, myc-KA2(T675A), and myc-KA2(T675E) protein were abundant following immunoprecipitation of GFP-GluR6 protein (Fig. 1C, IP lane), indicating that the wild-type and mutant KA2 subunits had formed multimeric receptors with GFP-GluR6 subunits. These results suggested that the absence of AMPA-evoked currents in heteromeric receptors was not due to compromised assembly of mutant KA2 subunits with GluR6.

In our initial physiology recordings we were not able to resolve whether glutamate-evoked currents in a subset of co-transfected cells arose from heteromeric GluR6/KA2(T675A) and GluR6/KA2(T675E) receptors or rather from homomeric GluR6 receptors. To determine if cells expressing both GluR6 and the KA2 mutants contained heteromeric kainate receptors on their plasma membrane, we assessed their localization using immunofluorescent techniques. COS-7 cells were co-transfected with GFP-GluR5–2b or GFP-GluR6 with an anti-GFP primary antibody under non-permeabilizing conditions, followed by permeabilization and labeling of intracellular myc-KA2 (or KA2 mutants) with an anti-myc antisera. Fig. 2A shows representative examples of the three-color analysis we performed in these experiments: green fluorescence arises from total GFP-GluR5–2b or GFP-GluR6 receptors, red from surface-GFP-tagged receptors, and cyan from intra- and extracellular myc-KA2 (originally acquired at long red wavelengths and false-colored for clarity). In cells expressing wild-type GFP-GluR5–2b/myc-KA2 and GFP-GluR6/myc-KA2, GFP-tagged receptors were abundant on the plasma membrane of co-transfected cells (Fig. 2A, center column). In contrast, cells co-transfected with myc-KA2(T675A) or myc-KA2(T675E) had significantly lower levels of detectable surface GFP-GluR5–2b or GFP-GluR6, and indeed most of the co-transfected cells imaged had no apparent receptor subunits on the plasma membrane. These results suggest that the altered KA2 subunit acts as dominant suppressor of forward trafficking in heteromeric kainate receptors and support the interpretation that the residual glutamate currents in patch clamp recordings arose from homomeric GluR6 receptors that did not contain a mutant KA2 subunit.

We also tested if mutation of the ligand binding site prevented surface expression of a homomeric KA2 subunit. As described previously (14), wild-type KA2 receptors are efficiently retained in the endoplasmic reticulum by virtue of an arginine-based retention/retrieval signal in the cytoplasmic tail. Alanine substitution of this motif in KA2-R/A mutants releases KA2 receptors to the plasma membrane (see Fig. 2B), but the receptors still do not gate currents in response to glutamate application (14). Mutation of Thr-675 in myc-KA2-R/A subunit eliminated surface expression of the homomeric receptors, as shown with surface-staining immunofluorescence for anti-myc epitopes in Fig. 2B (top row of images). Introduction of the Thr-675 mutations also prevented surface expression of a chimeric KA2 receptor containing the carboxyl-terminal domain of the GluR6 subunit (myc-KA2ΔR6, bottom row in Fig. 2B), which contains a forward trafficking determinant that efficiently drives plasma membrane localization of homomeric...
receptors (17). Thus ligand binding is a critical prerequisite for surface expression of both heteromeric and homomeric kainate receptors, even when the latter do not respond to glutamate application with channel gating.

The reduced surface expression was also observed in cell ELISA assays to quantitate surface expression of receptor protein (Fig. 3). Live COS-7 cells expressing GFP-GluR6 with myc-KA2 or myc-KA2 Thr-675 mutants were incubated with anti-GFP antisera to label surface receptors and a second antibody conjugated to horseradish peroxidase that generated a color reaction upon addition of substrate. GFP-GluR6/myc-KA2 cells exhibited robust surface expression of GFP epitopes (Fig. 3A), whereas surface expression of GFP-GluR6 was reduced by >90% when co-expressed with myc-KA2(T675A)- and GluR6/KA2(T675E)-expressing cells, because their desensitization rates were significantly slower than that of wild-type GluR6/KA2 receptors but not significantly different from GluR6 receptors (34).

We next tested if heteromeric kainate receptors expressed by cultured neurons also require an intact ligand site. There is currently no specific antibody that recognized extracellular epitopes of kainate receptor subunits, so we transfected GFP-GluR6 alone or in combination with myc-KA2, myc-KA2(T675A), or myc-KA2(T675E) cDNAs into cultured hippocampal neurons and localized receptor subunits using immunofluorescence imaging (Fig. 4). Live neurons were exposed to anti-GFP antibody before fixation and permeabilization, followed by anti-myc antibodies to localize myc-KA2-expressing neurons. As shown in the figure, GFP-GluR6 and GFP-GluR6/myc-KA2 receptors localized to the somatic and dendritic membranes (red panels in the top row of images) and internal compartments (green GFP fluorescence). In contrast, co-expression of GFP-GluR6 with myc-KA2(T675A) or myc-KA2(T675E) greatly reduced surface expression of GFP-GluR6-containing receptors (Fig. 4A, bottom two sets of images), as was observed in the transfected COS-7 cells. In addition to preventing surface expression of the heteromeric kainate receptors, myc-KA2(T675A) and myc-KA2(T675E) also redistributed intracellular GFP-GluR6 protein into a predominantly somatic compartment, with little staining evident in the dendritic processes, in contrast to GFP-GluR6/myc-KA2 receptors.
expressing neurons. To quantitate these changes in surface expression of GFP-GluR6 containing receptors, we measured the fluorescent intensities in the red (surface GFP-GluR6) and green (total GFP-GluR6) channels and expressed those values as a ratio (Fig. 4B). GFP-GluR6 and GFP-GluR6/myc-KA2 had relatively high red:green ratios (0.85 ± 0.03 and 0.68 ± 0.06, respectively, n = 7) compared with GFP-GluR6/myc-KA2(T675A) and GFP-GluR6/myc-KA2(T675E) (0.24 ± 0.04 and 0.23 ± 0.04, respectively, n = 8), indicating that the mutant KA2 subunits reduced proportional surface expression of GFP-GluR6 by ~65% in neurons.

To determine if elimination of glutamate binding in the KA2 mutants caused redistribution intracellularly, we co-localized myc-KA2-R/A and myc-KA2-R/A(T675E) with two markers for prominent organelles: protein di-sulfide isomerase (PDI), which is resident in the ER, and giantin, a cis- and medial-Golgi resident protein. As shown in Fig. 5, myc-KA2-R/A co-localized extensively in PDI-stained reticular structures characteristic of ER in COS-7 cells. Little overlap was observed with giantin, despite the surface localization of this homomeric receptor, suggesting that the proportion of total receptor contained within the Golgi was very small. This pattern of expression was similar for myc-KA2-R/A(T675E) (Fig. 5, right panels), which also co-localized with PDI but not with giantin. This analysis and similar immunofluorescent co-localization assays with heteromeric GFP-GluR6 and myc-KA2 receptors (data not shown) did not allow us to identify unequivocally the intracellular site of retention. We also attempted to assess maturation of myc-KA2-R/A and GFP-GluR6/myc-KA2 receptors using biochemical assays for resistance to endoglycosidase H but were unable to detect enzyme-resistant receptor protein (even in heteromeric subunit combinations that produced functional receptors). Additionally, we assessed whether kainate receptors were subject to sulfation, a post-translational modification dependent upon the Golgi apparatus that has been shown to modify the GluR2 AMPA receptor subunit (18). However, neither myc-KA2-R/A receptors or GFP-GluR6/myc-KA2 receptor subunits were sulfated in detectable quantities (data not shown), precluding use of this assay as a means of analyzing the site of retention of ligand-binding mutants. In summary, we conclude that the gross subcellular distribution of the KA2 receptor was not altered by elimination of ligand binding but
the precise site of retention remains unclear.

An increased rate of receptor degradation in principle could account for the reduction in surface expression of KA2-containing receptors. We tested this possibility in pulse-chase experiments to compare the degradation rate of homomeric myc-KA2 and Thr-675 mutant receptors (Fig. 6). Transfected HEK 293

Fig. 4. Expression of KA2 mutant subunits in cultured neurons reduces surface expression of GFP-GluR6 receptors. A, cultured hippocampal neurons were co-transfected with GFP-GluR6 and myc-KA2, myc-KA2(T675A), or myc-KA2(T675E) cDNAs at 3–4 days in vitro and maintained for another week in culture before imaging. Red images were surface GFP-detected with anti-GFP antibody under non-permeabilizing conditions, green images are of the total GFP fluorescence, and blue images are false-colored staining for anti-KA2 immunoreactivity after permeabilization of neurons. Co-expression with myc-KA2(T675A) or myc-KA2(T675E) markedly reduced the surface staining for anti-GFP and appeared to restrict intracellular GFP fluorescence to the soma and proximal processes. B, quantitation of the relative fluorescence intensity in the red and green channels as a measure of the relative expression of surface GFP-GluR6 versus total GFP-GluR6 receptors. Co-expression of GFP-GluR6 with myc-KA2 slightly reduced the surface expression of GFP antigen, whereas co-expression with myc-KA2(T675A) or myc-KA2(T675E) reduced GFP-GluR6 surface localization by ~4-fold.
cells were incubated in $^{35}$S-methionine for 30 min before chasing with media containing unlabeled methionine for 0–16 h, as indicated in Fig. 6. KA2 subunit protein was immunoprecipitated with anti-myc antibody and Western blots were performed as shown in the figure. Myc-KA2 appears as a double band of radiolabeled protein that decays rapidly after removal of $^{35}$S-methionine. Treatment of the samples with endoglycosidase H reduced the higher molecular weight protein to a similar size as the smaller band, confirming that the former represented an immature glycosylated form of the KA2 subunit.

**Fig. 5.** Kainate receptors lacking a ligand binding site are retained in the endoplasmic reticulum. Myc-KA2-R/A (left set of images) and myc-KA2-R/A(T675E) (right set of images) receptors were co-localized with protein disulfide isomerase (PDI), a marker for ER compartments and giantin, a cis- and medial-Golgi resident protein. Anti-myc staining for KA2 receptors is in red (top row), intracellular marker staining is in green (left, PDI and right, giantin in the center rows), and the overlay images are shown in the bottom row.

**Fig. 6.** Degradation rates are not changed in KA2 binding mutants. Pulse-chase assays were performed in myc-KA2, myc-KA2(T675A) and myc-KA2(T675E) receptor expressing cells. HEK QA cells were labeled metabolically for 30 min before chasing for the times indicated in the figure. Myc-KA2 and mutant subunits were immunoprecipitated with anti-myc antibody, electrophoresed on SDS-PAGE gels, and quantitated using phosphorimaging analysis. Band densities were normalized to the densities immediately following labeling (time point zero).
Ligand Binding Controls Kainate Receptor Trafficking

DISCUSSION

It has become clear recently that excitatory synaptic strength can be modulated by regulation of receptor trafficking and biosynthesis (1, 9). The carboxyl-terminal domains of ionotropic glutamate receptor subunits, which are cytoplasmic, are the primary site in the subunit proteins that contains critical determinants of subcellular and synaptic receptor localization. Recent studies have elucidated a number of cytoplasmic determinants of kainate receptor trafficking and biosynthesis, revealing that a diverse array of amino acid motifs play critical roles in the intracellular trafficking (14–17). In addition, it has been shown that AMPA and kainate receptors contain non-cytoplasmic determinants of subcellular localization. For AMPA receptors, these include the Q/R site in the pore-forming re-entrant loop (18, 24) and a short motif in the amino-terminal domain (35). As well, both AMPA and kainate receptors appear to require an intact ligand-binding domain for forward progression in the secretory pathway from the ER (25, 26), although it has been unclear how ligand binding operated as a permissive mechanism for forward trafficking.

Our results support this role for glutamate binding as a quality-control checkpoint in trafficking and surface expression of kainate receptors and clarify potential mechanisms underlying this regulation (25, 26). We found that homomeric and heteromeric kainate receptors lacking affinity for their endogenous ligand are sequestered intracellularly and that retention occurs even in receptors only partially composed of binding-deficient subunits. Dominant ER retention of receptors incorporating binding-deficient mutants occurred in both heterogeneous cell lines and cultured neurons. Mutant subunits were not compromised in their ability to co-assemble into multimeric receptors with wild-type receptor subunits nor were they degraded at a more rapid rate than wild-type KA2 subunits. These results suggest that each component subunit within a tetrameric glutamate receptor requires an intact binding site for transit forward from the ER.

Dominant retention by the mutant KA2 subunits could be accounted for mechanistically in at least three ways: mutations caused misfolding of the subunit protein sufficient to activate quality-control systems in the ER, binding-related structural changes necessary for forward trafficking were prevented, or channel activation and gating equivalent to agonist-evoked steady-state currents were necessary for forward transit through the secretory pathway. We believe that misfolding is unlikely, because no change was detected in the degradation rate of wild-type and mutant KA2 subunit proteins, suggesting elimination of the ligand binding site did not activate the ER-associated degradation quality control system or other molecular chaperones that would promote more rapid elimination of aberrant receptors. This result is qualitatively similar to that observed for Q/R site-edited GluR2 AMPA receptor subunits, which reside stably in the ER as monomers or dimers and are not subject to accelerated degradation (18, 24). However, GluR2(R) subunits are substantially longer-lived in the ER compared with KA2 subunits, which exhibited a fairly rapid turnover rate of a couple of hours in our experiments. This rate of degradation is also significantly faster than that of homomeric GluR6 receptors, which are stable at least 4 h when trapped in the ER by brefeldin treatment before appreciable degradation occurs (17). This difference in rates suggests that assembly of heteromeric GluR6/KA2 receptors might be rate-limited by the degradation of KA2 subunits, which could in part explain the common observation that mixtures of homomeric GluR6 and heteromeric GluR6/KA2 receptors are formed despite the preponderance of KA2 cDNA in transfection conditions like those used in our study. In summary, these data demonstrate that the homomeric form of KA2 subunits, which do not normally traffic to the plasma membrane, are targeted by quality control systems in the ER but mutation of the glutamate binding site does not alter this turnover rate.

Because altered degradation does not appear to play a role in the retention mechanism, we conclude that glutamate binding to ER-resident receptors provides a read-out of the receptor functional state as a mechanism for biosynthetic quality control. We attempted to test this hypothesis more directly by incubating kainate receptor-expressing COS-7 cells in the antagonist CNQX for 24 h prior to cell ELISA assays, but this assay did not result in reductions in surface expression of the receptors (data not shown). These negative data are difficult to interpret, however, because it is possible that CNQX did not penetrate the critical intracellular compartment containing nascent receptors binding glutamate. Additionally, CNQX is a competitive antagonist and might have been present in sufficient concentration to completely displace endogenous glutamate binding. The concentration of glutamate in ER is unknown, but relatively low concentrations induce conformational changes in glutamate receptor structure correlated with gating and desensitization; for example, application of 1–5 μM glutamate effectively desensitized kainate (36) and AMPA and NMDA receptors (37). Thus, it would not require particularly high levels of ambient ER glutamate to elicit structural changes in fully assembled receptors.

Glutamate binding to receptors in ER is predicted to have two primary consequences: desensitization-related structural changes in the “extracellular” domains of the subunits and equilibrium cation currents through the receptor channels. Our results are most consistent with the former as a mechanism for quality control mediated by ligand binding. Desensitization of ionotropic glutamate receptors has been modeled structurally from crystallographic analysis of the GluR2 subunit ligand-binding domain as a destabilization of the subunit interface domains (38). This rearrangement in principle could expose chaperone binding sites on the receptor that are necessary for further transit in the biosynthetic pathway. Alternatively, partially assembled subunits might contain ER retention/retrieval motifs that are masked upon tetramerization and glutamate binding. We favor structural rearrangements as being relevant to quality control rather than ion permeation, because the T675E and T675A binding site mutations cause retention of homomeric myc-KA2-R/A or myc-KA2A6 receptors. These receptors bind glutamate and traffic to the plasma membrane, unlike wild-type KA2 receptors, but nonetheless do not gate currents in response to glutamate (14), and thus it is unlikely that ion permeation occurs in the ER. This hypothesis is consistent with previous observations that mutations in gating domains also causes retention of homomeric GluR6 subunits (26); such mutations might occlude necessary conformational rearrangements necessary for chaperone recognition.

It is also possible that conformational changes associated with ligand binding are necessary for efficient assembly of tetrameric receptors, and that mutation of ligand binding or gating sites occludes oligomerization. AMPA receptors assemble as dimer of dimers, and this process is regulated by the...
amino acid residing at the Q/R site in GluR2 subunits (18, 24). It is not clear if kainate receptors, which exhibit much greater variability than GluR2 in the degree of their Q/R site RNA editing in situ (39, 40), are subject to the same rules of oligomerization, but domain-swapping experiments suggested that this might be the case (41). Our co-immunoprecipitations of GFP-GluR6 with mutant KA2 subunits suggest that, despite retention, at least one subunit of KA2 can assemble with a GluR6 subunit in the ER, although we do not know if tetrameric receptors are formed. Similarly, myc-KA2-R/A receptors traffic forward to the plasma membrane (14), but because these receptors remain insensitive to glutamate we cannot be sure they are tetrameric rather than monomeric or dimeric receptor subunits.

In summary, we have identified an important quality-control mechanism that controls functional expression of ionotropic glutamate receptors through ligand-induced conformational changes in the ER. Future correlation of binding and gating mutations with changes in intracellular trafficking and assembly will facilitate an understanding of the critical elements of this regulatory process.

REFERENCES
1. Bredt, D. S., and Nicoll, R. A. (2003) Neuron 40, 361–379
2. Perez-Otano, I., and Ehlers, M. D. (2004) Neurosignals 13, 175–189
3. Hayashi, Y., Shi, S. H., Esteban, J. A., Piccini, A., Poncer, J. C., and Malinow, R. (2000) Science 287, 2262–2267
4. Ehlers, M. D. (2000) Neuron 28, 279–289
5. Man, H. Y., Lin, J. W., Ju, W. H., Ahmadian, G., Liu, L., Becker, L. E., Sheng, M., and Wang, Y. T. (2000) Neuron 25, 649–662
6. Carroll, R. C., Lissin, D. V., von Zastrow, M., Nicoll, R. A., and Malenka, R. C. (1999) Nat. Neurosci. 2, 454–460
7. Grosshans, D. R., Clayton, D. A., Coultrap, S. J., and Browning, M. D. (2002) Nat. Neurosci. 5, 27–33
8. Nong, Y., Huang, Y. Q., Ju, W., Kalia, L. V., Ahmadian, G., Wang, Y. T., and Salter, M. W. (2003) Nature 422, 302–307
9. Prybylowski, K., and Wenthold, R. J. (2004) J. Biol. Chem. 279, 9673–9676
10. Kidd, P. L., and Isaac, J. T. (1999) Nature 400, 569–573
11. Ghetta, A., and Heinemann, S. F. (2000) J. Neurosci. 20, 2766–2773
12. Scott, D. B., Blanpied, T. A., Swanson, G. T., Zhang, C., and Ehlers, M. D. (2001) J. Neurosci. 21, 3063–3072
13. Standley, S., Roche, K. W., McCullum, J., Sans, N., and Wenthold, R. J. (2000) Neuron 28, 887–898
14. Ren, Z., Riley, N. J., Garcia, E. P., Sanders, J. M., Swanson, G. T., and Marshall, J. (2003) J. Neurosci. 23, 6608–6616
15. Ren, Z., Riley, N. J., Needlemen, L. A., Sanders, J. M., Swanson, G. T., and Marshall, J. (2003) J. Biol. Chem. 278, 52700–52709
16. Jaskolski, F., Coussein, F., Nagarajan, N., Normand, E., Rosenmund, C., and Mulle, C. (2004) J. Neurosci. 24, 2506–2515
17. Yan, S., Sanders, J. M., Xu, J., Zhu, Y., Contractor, A., and Swanson, G. T. (2004) J. Neurosci. 24, 679–691
18. Greger, I. H., Khatri, L., and Ziff, E. B. (2002) Neuron 34, 759–772
19. Hawkins, L. M., Prybylowski, K., Chang, K., Mousseau, C., Stephenson, F. A., and Wenthold, R. J. (2004) J. Biol. Chem. 279, 28903–28910
20. Matsuda, I., and Mishina, M. (2000) Biochem. Biophys. Res. Commun. 275, 565–571
21. Ellgaard, L., and Helenius, A. (2003) Nat. Rev. Mol. Cell. Biol. 4, 181–191
22. Burnashev, N., Monyer, H., Seeburg, P. H., and Sakmann, B. (1992) Neuron 8, 149–158
23. Swanson, G. T., Kamboj, S. K., and Cull-Candy, S. G. (1997) J. Neurosci. 17, 58–69
24. Greger, I. H., Khatri, L., Kong, X., and Ziff, E. B. (2003) Neuron 40, 763–774
25. Grunwald, M. E., and Kaplan, J. M. (2003) Neuronpharmacology 45, 768–776
26. Fleck, M. W., Cornell, E., and Mah, S. J. (2003) J. Neurosci. 23, 1219–1227
27. Herb, A., Burnashev, N., Werner, P., Sakmann, B., Wisden, W., and Seeburg, P. H. (1992) Neuron 8, 775–785
28. Schiffer, H. H., Swanson, G. T., and Heinemann, S. F. (1997) Neuron 19, 1141–1146
29. Murthy, V. N., Sejnowski, T. J., and Stevens, C. F. (1997) Neuron 18, 599–612
30. Armstrong, N., and Gouaux, E. (2000) Neuron 28, 165–181
31. Anson, L. C., Chen, P. E., Wyllie, D. J. A., Colquhoun, D., and Schoepfer, R. (1996) J. Neurosci. 16, 581–589
32. Pas, Y., Eisenstein, M., Medevieville, F., Teichberg, V. I., and Devillers-Thiéry, A. (1996) Neuron 17, 979–990
33. Swanson, G. T., Green, T., and Heinemann, S. F. (1998) Mol. Pharmacol. 53, 942–949
34. Swanson, G. T., Gereau, IV, R. W., Green, T., and Heineman, S. F. (1997) Neuron 19, 913–926
35. Xia, H., von Zastrow, M., and Malenka, R. C. (2002) J. Biol. Chem. 277, 47705–47709
36. Paternain, A. V., Rodriguez-Moreno, A., Villarreal, A., and Lerma, J. (1998) Neuropharmacology 37, 1249–1259
37. Zorumski, C. F., Mennerick, S., and Que, J. (1996) J. Physiol. 494, 465–477
38. Sun, Y., Olson, R., Horning, M., Armstrong, N., Mayer, M., and Gouaux, E. (2002) Nature 417, 245–253
39. Bernard, A., Ferhat, L., Dass, F., Charton, G., Represa, A., Ben-Ari, Y., and Khrestchatisky, M. (1999) Eur. J. Neurosci. 11, 604–616
40. Bernard, A., and Khrestchatisky, M. (1994) J. Neurochem. 62, 2057–2060
41. Ayalon, G., and Stern-Bach, Y. (2001) Neuron 31, 103–113
Ligand Binding Is a Critical Requirement for Plasma Membrane Expression of Heteromeric Kainate Receptors
Lokanatha Valluru, Jian Xu, Yongling Zhu, Sheng Yan, Anis Contractor and Geoffrey T. Swanson

J. Biol. Chem. 2005, 280:6085-6093.
doi: 10.1074/jbc.M411549200 originally published online December 6, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M411549200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 41 references, 14 of which can be accessed free at http://www.jbc.org/content/280/7/6085.full.html#ref-list-1