0026-895X/03/6403-703–706$7.00
MOLECULAR PHARMACOLOGY
Copyright © 2003 The American Society for Pharmacology and Experimental Therapeutics
Mol Pharmacol 64:703–706, 2003

Vol. 64, No. 3
2472/1085114
Printed in U.S.A.

Stargazin Differentially Controls the Trafficking of α-Amino-3-hydroxyl-5-methyl-4-isoxazolepropionate and Kainate Receptors

LU CHEN, ALAA EL-HUSSEINI, SUSUMU TOMITA, DAVID S. BREDT, and ROGER A. NICOLL

Departments of Cellular and Molecular Pharmacology (L.C., R.A.N.) and Physiology (A.E.-H., S.T., D.S.B., R.A.N.), University of California at San Francisco, California; and Kinsmen Laboratory, Department of Psychiatry and the Brain Centre, University of British Columbia, Vancouver, British Columbia (A.E.-H.)

Received February 23, 2003; accepted May 21, 2003

This article is available online at http://molpharm.aspetjournals.org

ABSTRACT

Synaptic plasticity at excitatory synapses in the brain is largely achieved by rapid changes in the number of synaptic α-amino-3-hydroxyl-5-methyl-4-isoxazolepropionate (AMPA) receptors. Stargazin, a membrane protein that interacts with AMPA receptors, is believed to play a pivotal role in trafficking AMPA receptors to the plasma membrane and targeting them to the synapse. However, it is unclear whether the trafficking of kainate receptors, which are structurally very similar to AMPA receptors, is also dependent on stargazin. Here we show that in both cerebellar granule cells and in *Xenopus laevis* oocytes expression system, surface delivery of kainate receptor is independent of stargazin. These results suggest that stargazin action is highly selective for AMPA receptors.

Glutamate, the transmitter released from excitatory synapses in the brain, acts on three subtypes of ionotropic receptors: AMPA, NMDA, and kainate receptors. Synaptic AMPA receptors, in contrast to NMDA receptors, are highly mobile (Song et al., 1998; Luscher et al., 1999; Luthi et al., 1999; Noel et al., 1999; Ziff, 1999; Sheng, 2001; Malinow and Malenka, 2002), and changes in the number of synaptic AMPA receptors play an essential role in synaptic plasticity (Luscher et al., 1999; Daw et al., 2000; Malinow et al., 2000; Lu et al., 2001). We showed previously (Chen et al., 2000) that the delivery of AMPA receptors to the surface of cerebellar granule cells requires stargazin, the four-pass transmembrane protein defective in the ataxic stargazer mouse. On the other hand, the trafficking of NMDA receptors in these cells is independent of stargazin, highlighting the fundamental difference in the mechanisms involved in the delivery of these two subtypes of glutamate receptors to the membrane surface. In contrast to the abundant literature on the trafficking of AMPA and NMDA receptors, little is known about kainate receptor trafficking. In particular, a role for stargazin in this process has not been addressed. Because kainate receptors are structurally very similar to AMPA receptors, such information will provide important insight into the selectivity of the action of stargazin. To address this issue, we used the *Xenopus laevis* oocyte expression system, which allowed us to measure quantitatively the influence of stargazin on the surface expression of glutamate receptors. This system also allowed us to test whether stargazin effects on AMPA receptor trafficking can occur without other neuron-specific components.

Materials and Methods

Constructs. Stargazin, γ-1, and glutamate receptor subunits were subcloned into pGEM-HE vector (a gift from Lily Jan, University of California San Francisco). In vitro transcription of cRNAs was done using AmpliScribe T7 transcription kit (Epicentre Technologies, Madison, WI).

Granule Cell Culture and Electrophysiology. Stargazer cerebellar granule cell cultures were prepared from 5- to 7-day-old mouse pups as described previously (Chen et al., 2000). Cells were kept in minimum essential medium (5.3 K^+) (Invitrogen, Carlsbad, CA) supplemented with glucose (0.5%), transferrin (0.1 mg/ml), insulin (0.025 mg/ml), glutamine (2 mM), cytosine arabinoside (4 μM), and 10% heat-inactivated fetal calf serum (Invitrogen). Tail samples

This work was supported by National Institutes of Health grant P50-MH63981 (to R.A.N. and D.S.B.) and by the Howard Hughes Medical Institute, the Christopher Reeve Paralysis Foundation, and the Human Frontier Science Program (to D.S.B.). D.S.B. is an established investigator of the American Heart Association and a member of the Silvio Conte Center for Neuroscience Research. R.A.N. is a member of the Keck Center for Integrative Neuroscience and the Silvio Conte Center for Neuroscience Research. L.C. is supported by National Research Service Award postdoctoral fellowship F32-MH65112.

ABBREVIATIONS: AMPA, α-amino-3-hydroxyl-5-methyl-4-isoxazolepropionate; NMDA, *N*-methyl-D-aspartate; ConA, concanavalin A; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; NHS-SS-biotin, *N*-hydroxysuccinimide bound to biotin; ANOVA, analysis of variance; KA, kainate; GYKI 53655, 1-(4-aminophenyl)-3-methylcarbamyl-4-methyl-7,8-methylenedioxy-3,4-dihydro-5H-2,3-benzodiazepine.

from individual pups were used for genotyping with described primers (Letts et al., 1998).

Whole-cell patch-clamp recordings were made at room temperature using 3 to 7 MΩ patch pipettes filled with an internal solution containing 140 mM CsCl, 2 mM $MgCl_2$, 5 mM EGTA, 10 mM HEPES, 0.3 mM Na_3-GTP, and 4 mM Na_2-ATP, pH 7.35. Cultures were continuously superfused with external solution containing 119 mM NaCl, 26 mM $NaHCO_3$, 2.5 mM KCl, 10 mM glucose, 2.5 mM $CaCl_2$, 1.3 mM $MgCl_2$, 1 mM NaH_2PO_4, 0.1 mM picrotoxin, and 1 mM tetrodotoxin. Cells were incubated in concanavalin A (ConA) (0.25 mg/ml) for 15 to 20 min before being transferred to the recording chamber. Fast agonist (100 μM KA) application was achieved by gravity feeding as described previously (Lester and Jahr, 1992).

Oocyte Electrophysiology. Defolliculated *X. laevis* oocytes were injected with 0.1 to 1 ng of cRNA as described previously (Caterina et al., 1997). Two-electrode voltage-clamp analysis (E_h = −60 mV) was carried out 1 to 5 days after injection at room temperature. Frog Ringer's solution contained 90 mM NaCl, 1.0 mM KCl, 1.5 mM $BaCl_2$, 1.0 mM $MgCl_2$, and 10 mM HEPES, pH 7.6. Agonist glutamate (100 μM) was bath-applied together with cyclothiazide (100 μM) to block the desensitization of AMPA receptors.

Biotinylation of Cell Surface Proteins. Oocytes injected with GluR1 cRNA or GluR1 plus stargazin cRNAs were incubated with 1.5 mg/ml sulfo NHS-SS-biotin in ND96 (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, and 5 mM HEPES) on ice for 30 min, then washed three times with cold ND96 containing 50 mM glycine. Membranes were prepared, and cell surface-biotinylated proteins were precipitated with streptavidin-agarose (Pierce Chemical, Rockford, IL) and detected by Western blotting. Rabbit polyclonal antibody to GluR1 (Chemicon International, Temecula, CA) was used for GluR1 protein detection. The total is 3% of the amount that is loaded in surface.

Results

Oocytes were injected with cRNA for various glutamate receptor subunits and stargazin, and the currents evoked by glutamate were measured with two-electrode voltage clamp 1 to 4 days after injection. The desensitization of AMPA receptors was blocked by cyclothiazide. Coexpression of stargazin with GluR1 resulted in a dramatic enhancement of the glutamate-evoked currents (Fig. 1a). On average, the currents were enhanced approximately 10-fold (normalized, 11.0 ± 0.7-fold). Stargazin (γ2) is a member of a family of four transmembrane domain proteins, termed γ1–8. γ1, which is a calcium-channel subunit and shows weak homology with stargazin, was unable to mimic the actions of stargazin, indicating a functional heterogeneity in this family of proteins (normalized, 1.1 ± 0.1-fold) (Fig. 1b) and demonstrating the specificity of the stargazin effects on GluR1 currents. Dose-response curves revealed that the enhancement was largely caused by an increase in the maximum response, because there was little change in the affinity of the response (Fig. 1c). This finding is consistent with an increase in the number of receptors on the surface. We carried out biotinylation assays to verify that stargazin enhances the number of surface receptors. As shown in Fig. 1d, stargazin had no obvious effect on the total amount of GluR1, but it caused a substantial increase in the amount of surface GluR1 (surface-to-total ratio is increased by 7.5 ± 2.4-fold in stargazin group, n = 3). Stargazin also enhanced the currents evoked by homomeric GluR2 and GluR4 receptors (data not shown). AMPA receptors (GluR1–GluR4) are normally expressed in neurons as heteromers. We therefore examined the ability of stargazin to influence the delivery of GluR1 plus GluR2 and GluR2 plus GluR4 to the membrane surface. It was shown before that when two AMPA receptor subunits are coexpressed in oocytes, they preferentially form heteromeric receptor complexes, indicated by their current-voltage relationship (Ayalon and Stern-Bach, 2001). Therefore, we coinjected an equal amount of GluR1 plus GluR2 or GluR4 plus GluR2 cRNAs to examine stargazin's effect on heteromeric AMPA receptor complexes. Stargazin retained its ability to enhance glutamate-evoked currents (GluR1 plus GluR2: 5.9 ± 1.0-fold; GluR2 plus GluR4: 5.0 ± 0.5-fold) (Fig. 2).

To examine whether stargazin controls kainate receptor delivery to the membrane surface, we measured kainate-evoked responses in cultured cerebellar granule cells, which also express this class of receptor (Savidge et al., 1999). Although there are multiple kainate receptor subunits expressed in the cerebellum (Porter et al., 1997), the cerebellar granule cells express GluR6 and KA2 at high levels (Egebjerg et al., 1991; Herb et al., 1992). We confirmed that in wild-type or heterozygous granule cells, kainate evoked responses with pharmacological properties that were consistent with kainate receptors (Fig. 3, a and b). Thus, in the presence of the AMPA receptor antagonist GYKI 53655, kainate only evoked currents in cells preincubated in ConA, which selectively blocks the desensitization of kainate receptors. In addition, the response was blocked by the non-NMDA receptor antagonist CNQX. We found no difference in the size of the kainate receptor-mediated responses in heterozygous neurons or in neurons lacking stargazin (+/stg, 29.0 ± 3.2 pA; stg/stg, 25.7 ± 1.7 pA; no ConA, 3.3 ± 1.1 pA; CNQX, 4.0 ± 2.7 pA)

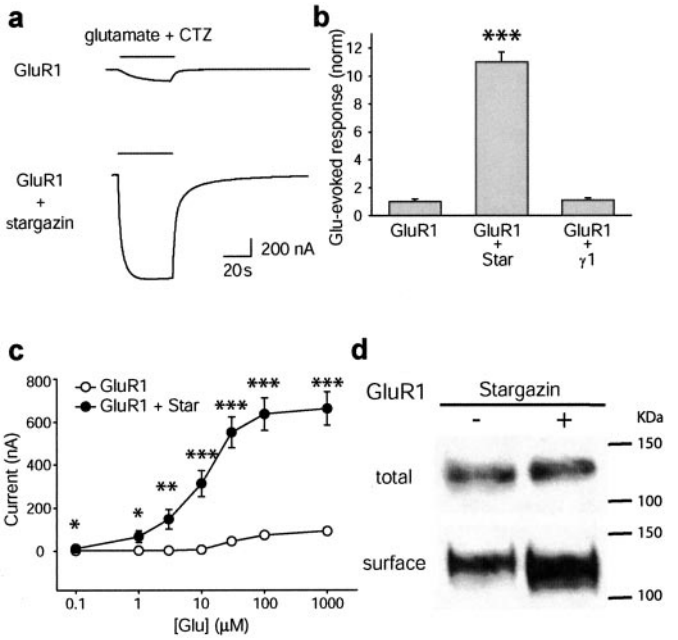

Fig. 1. Stargazin enhances GluR1 surface expression in oocytes. a, examples of glutamate-evoked currents in oocytes expressing GluR1 alone or GluR1 with stargazin. CTZ, cyclothiazide. b, in oocytes that express stargazin, GluR1-mediated currents were greatly enhanced (n = 7; ***, $p < 0.001$, single-factor ANOVA). Calcium-channel subunit γ-1 failed to enhance GluR1-mediated currents (n = 7; $P > 0.5$). c, the enhancement of GluR1-mediated current cannot be explained by changes in agonist affinity. Even with a saturating dose of glutamate (1 mM), the GluR1 plus stargazin group still had a significantly larger current (n = 10; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). d, surface biotinylation assay showed that GluR1 surface expression is greatly enhanced when it is coexpressed with stargazin (n = 3; $P < 0.05$).

(Fig. 3b). To examine in more detail for a possible interaction between stargazin and kainate receptors, we expressed in oocytes the kainate receptor subunit GluR6 either alone or with stargazin (Fig. 3c). Stargazin had no effect on the magnitude of the kainate receptor-evoked currents recorded over a 3-day expression period.

Discussion

In conclusion, we found that kainate receptor-mediated responses, in striking contrast to AMPA receptor-mediated responses, are normal in cerebellar granule cells from stargazin-deficient mice. To study this differential regulation more quantitatively, we used a simple assay system, namely the *X. laevis* oocyte. Stargazin was found to cause a 10-fold enhancement in AMPA receptor-mediated responses, whereas the kainate receptor-mediated responses were entirely independent of regulation by stargazin. We used the GluR6 kainate subunit in oocyte experiments because it is the most abundantly expressed subunit in cerebellar granule cells (Egebjerg et al., 1991; Herb et al., 1992). The normal kainate receptor-mediated response in stargazer cerebellar granule cells indicates that GluR6 and KA2 trafficking are stargazin-independent. However, we can not formally exclude the possibility that stargazin may interact with other kainate receptor subunits that are not expressed in cerebellar granule cells. Although similar in their response to glutamate, kainate receptors and AMPA receptors have quite different subcellular distributions. For example, at hippocampal mossy fiber −CA3 synapses, kainate receptors are found both pre- and postsynaptically (Kamiya, 2002), whereas AMPA receptors are localized only at the postsynaptic site. This suggests that different molecular mechanisms are used for kainate receptor trafficking. Given the close structural similarity of these two groups of receptors, it will be of interest to determine the specific structural requirement for this differential synaptic trafficking.

Previous studies have identified numerous protein partners for AMPA receptors that apparently regulate receptor clustering and retention at synapses (Ziff, 1999; Sheng, 2001; Malinow and Malenka, 2002). However, stargazin differs from other AMPA receptor-interacting proteins in several important ways. First, stargazin is the only known transmembrane protein to interact with AMPA receptors. Second, results here show that stargazin, as opposed to other AMPA receptor-binding proteins (Ziff, 1999; Sheng, 2001; Malinow and Malenka, 2002), directly regulates multiple GluR subunits—those with long cytosolic tails (GluR1 and GluR4), and those with short tails (GluR2). Finally, we demonstrate that stargazin enhances surface trafficking of AMPA receptors in a simplified system that lacks structural components of the synapse. Such direct regulation has not been reported previously for other AMPA receptor-binding proteins. These data may suggest that stargazin plays the primary role in surface targeting of AMPA receptors and that the additional protein interactions with AMPA receptors modulate and differentially anchor the AMPA receptor/stargazin complex. Future studies of these interactions in simplified systems—such as oocytes—should help clarify how stargazin and numerous cytosolic AMPA receptor-binding proteins regulate receptor trafficking and associated synaptic plasticity.

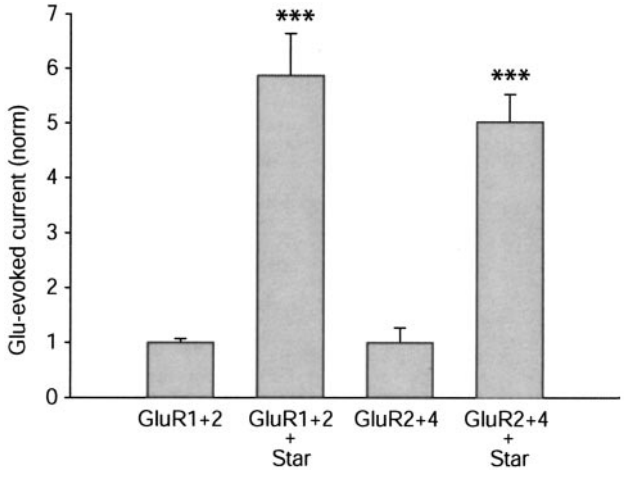

Fig. 2. In oocytes in which an equal amount of two AMPA receptor subunit cRNAs are injected (GluR1 + GluR2 or GluR4 + GluR2), stargazin enhances heteromeric AMPA receptor surface expression (GluR1 + GluR2, $n = 4$, ***, $p < 0.001$; GluR2 + GluR4, $n = 5$, ***, $p < 0.001$).

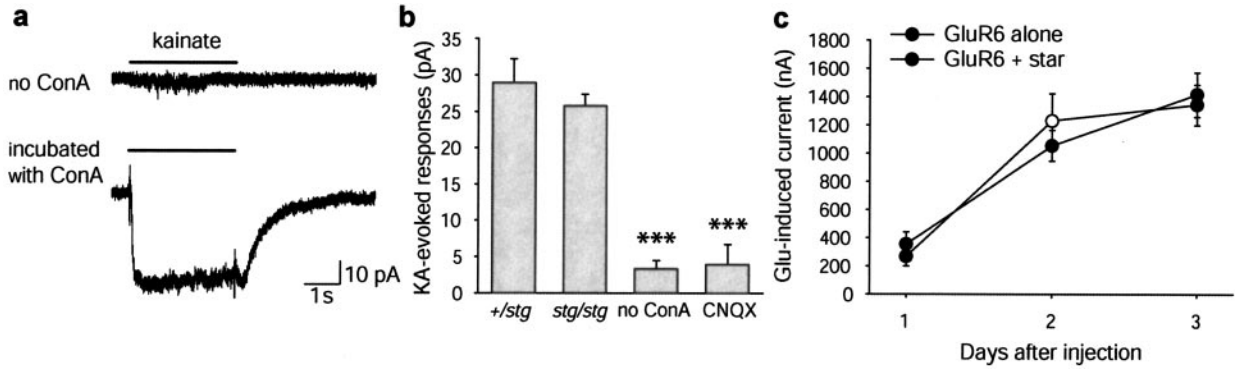

Fig. 3. Kainate receptor surface expression is stargazin-independent. a, wild-type cerebellar granule cell responses to kainate (100 μM) in the presence of GYKI 53655 (50 μM) require incubation of ConA (0.25 mg/ml). Application of kainate (100 μM) to cells incubated in ConA for 15 min evoked an inward current. This pharmacological profile demonstrates that functional KA receptors are expressed on cerebellar granule cell surface. b, stargazer mutant granule cells exhibit normal KA receptor-mediated responses to kainate (+/stg, $n = 8$; stg/stg, $n = 9$; $P > 0.1$, single-factor ANOVA), which can be blocked by CNQX (10 μM) (***, $P < 0.001$). c, when expressed in oocytes, KA receptor subunit GluR6 formed functional homomeric receptors and their trafficking to the plasma membrane is not enhanced by coexpression of stargazin (GluR6 alone: $n = 7$, GluR6 + stargazin: $n = 8$; group × day interaction, $P > 0.5$, ANOVA).

Acknowledgments

We thank Dr. David Julius for providing us with the oocyte recording facilities and Dr. Marilia Guimaraes for technical assistance in oocyte preparation.

References

Ayalon G and Stern-Bach Y (2001) Functional assembly of AMPA and kainate receptors is mediated by several discrete protein-protein interactions. *Neuron* **31:**103–113.

Caterina MJ, Schumacher MA, Tominaga M, Rosen TA, Levine JD, and Julius D (1997) The capsaicin receptor: a heat-activated ion channel in the pain pathway. *Nature (Lond)* **389:**816–824.

Chen L, Chetkovich DM, Petralia RS, Sweeney NT, Kawasaki Y, Wenthold RJ, Bredt DS, and Nicoll RA (2000) Stargazin regulates synaptic targeting of AMPA receptors by two distinct mechanisms. *Nature (Lond)* **408:**936–943.

Daw MI, Chittajallu R, Bortolotto ZA, Dev KK, Duprat F, Henley JM, Collingridge GL, and Isaac JT (2000) PDZ proteins interacting with C-terminal GluR2/3 are involved in a PKC-dependent regulation of AMPA receptors at hippocampal synapses. *Neuron* **28:**873–886.

Egebjerg J, Bettler B, Hermans-Borgmeyer I, and Heinemann S (1991) Cloning of a cDNA for a glutamate receptor subunit activated by kainate but not AMPA. *Nature (Lond)* **351:**745–748.

Herb A, Burnashev N, Werner P, Sakmann B, Wisden W, and Seeburg PH (1992) The KA-2 subunit of excitatory amino acid receptors shows widespread expression in brain and forms ion channels with distantly related subunits. *Neuron* **8:**775–785.

Kamiya H (2002) Kainate receptor-dependent presynaptic modulation and plasticity. *Neurosci Res* **42:**1–6.

Lester RA and Jahr CE (1992) NMDA channel behavior depends on agonist affinity. *J Neurosci* **12:**635–643.

Letts VA, Felix R, Biddlecome GH, Arikkath J, Mahaffey CL, Valenzuela A, Bartlett FS 2nd, Mori Y, Campbell KP, and Frankel WN (1998) The mouse stargazer gene encodes a neuronal Ca2+-channel gamma subunit. *Nat Genet* **19:**340–347.

Lu W, Man H, Ju W, Trimble WS, MacDonald JF, and Wang YT (2001) Activation of synaptic NMDA receptors induces membrane insertion of new AMPA receptors and LTP in cultured hippocampal neurons. *Neuron* **29:**243–254.

Luscher C, Xia H, Beattie EC, Carroll RC, von Zastrow M, Malenka RC, and Nicoll RA (1999) Role of AMPA receptor cycling in synaptic transmission and plasticity. *Neuron* **24:**649–658.

Luthi A, Chittajallu R, Duprat F, Palmer MJ, Benke TA, Kidd FL, Henley JM, Isaac JT, and Collingridge GL (1999) Hippocampal LTD expression involves a pool of AMPARs regulated by the NSF-GluR2 interaction. *Neuron* **24:**389–399.

Malinow R, Mainen ZF, and Hayashi Y (2000) LTP mechanisms: from silence to four-lane traffic. *Curr Opin Neurobiol* **10:**352–357.

Malinow R and Malenka RC (2002) AMPA receptor trafficking and synaptic plasticity. *Annu Rev Neurosci* **25:**103–126.

Noel J, Ralph GS, Pickard L, Williams J, Molnar E, Uney JB, Collingridge GL, and Henley JM (1999) Surface expression of AMPA receptors in hippocampal neurons is regulated by an NSF-dependent mechanism. *Neuron* **23:**365–376.

Porter RH, Eastwood SL, and Harrison PJ (1997) Distribution of kainate receptor subunit mRNAs in human hippocampus, neocortex and cerebellum and bilateral reduction of hippocampal GluR6 and KA2 transcripts in schizophrenia. *Brain Res* **751:**217–231.

Savidge JR, Sturgess NC, Bristow DR, and Lock EA (1999) Characterisation of kainate receptor mediated whole-cell currents in rat cultured cerebellar granule cells. *Neuropharmacology* **38:**375–382.

Sheng M (2001) Molecular organization of the postsynaptic specialization. *Proc Natl Acad Sci USA* **98:**7058–7061.

Song I, Kamboj S, Xia J, Dong H, Liao D, and Huganir RL (1998) Interaction of the N-ethylmaleimide-sensitive factor with AMPA receptors. *Neuron* **21:**393–400.

Ziff EB (1999) Recent excitement in the ionotropic glutamate receptor field. *Ann NY Acad Sci* **868:**465–473.

Address correspondence to: Dr. Roger A. Nicoll, Department of Cellular and Molecular Pharmacology, University of California at San Francisco, San Francisco, CA 94143. E-mail: nicoll@cmp.ucsf.edu