Stargazin Interaction with α-Amino-3-hydroxy-5-methyl-4-isoxazole Propionate (AMPA) Receptors Is Critically Dependent on the Amino Acid at the Narrow Constriction of the Ion Channel*

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The subunit GluR2 of the α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) subfamily of ionotropic glutamate receptors (GluRs) features a single amino acid at the narrow constriction of the pore loop that is altered from glutamine to arginine by RNA editing. This so-called Q/R site has been shown to play an important role in the determination of the electrophysiological properties of AMPA receptor complexes as well as of trafficking to the plasma membrane. The protein stargazin has also been shown to modulate electrophysiological properties and trafficking to the plasma membrane of AMPA receptors. In this study we examined via a series of mutants of the Q/R site of the AMPA receptor GluR1 whether the amino acid at this position has any influence on the modulatory effects mediated by stargazin. To this end, we analyzed current responses of Q/R site mutants upon application of glutamate and kainate and determined the amount of mutant receptor protein in the plasma membrane in Xenopus oocytes. Desensitization kinetics of several mutants were analyzed in HEK293 cells. We found that the stargazin-mediated decrease in receptor desensitization, the slowing of desensitization kinetics, and the kainate efficacy were all dependent on the amino acid at the Q/R site, whereas the stargazin-mediated increase in trafficking toward the plasma membrane remained independent of this amino acid. We propose that the Q/R site modulates the interaction of stargazin with the transmembrane domains of AMPA receptors via an allosteric mechanism and that this modulation leads to the observed differences in the electrophysiological properties of the receptor.

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The majority of the excitatory synaptic transmission in the mammalian central nervous system is mediated by AMPA4 receptors (AMPARs). These receptors assemble in the endoplasmic reticulum (ER) either as homo- or as heterotetramers (1) of the subunits GluR1 through GluR4. mRNA coding for GluR2 undergoes a posttranscriptional modification termed “editing” that alters a codon for a glutamine residue (Q) in the pore region into a codon for arginine (R). This position is called the Q/R editing site, or Q/R site (2). Receptor complex assembly is believed to take place via a two-step mechanism in which interaction of the N-terminal domains leads to the formation of dimers followed by the assembly of such dimers into tetramers (6). This tetramerization is dependent on the amino acid at the Q/R site (7). In synapses, AMPARs were shown to anchor themselves by binding to several PDZ domain-containing proteins such as GRIP, PICK-1, or SAP-97 (8–10). The insertion and removal (cycling) of AMPARs in the PSD is mediated by several proteins in an activity-dependent manner (11). One of these regulatory proteins is stargazin, which is disrupted in the stargazer mouse and was originally characterized as a homologue of the γ1 subunit of voltage-dependent calcium channels (12). The stargazer mouse lacks AMPAR-mediated responses in cerebellar granule cells, which can be rescued by overexpression of stargazin (13). Stargazin (γ2) belongs to the family of transmembrane AMPAR regulatory proteins (TARPs), which include the additional members γ3, γ4, and γ8. These proteins are also able to rescue the AMPAR-mediated response to glutamate application in cerebellar granule cells of the stargazer mouse and show a distinct expression pattern in the brain (13). Stargazin has been demonstrated to support receptor trafficking from the ER to the plasma membrane (14), and to stabilize AMPARs in the PSD by interaction with PSD-95 and n-PIST (15, 16). Further-
more, stargazin has been shown to modulate the electrophysiological properties of AMPARs by slowing down and decreasing desensitization, slowing down deactivation, decreasing the EC50 values for glutamate and kainate, increasing the probability of reaching the maximum conductance level, and increasing the mean burst length of the channel as well as the kainate efficacy (14, 17, 18). Here, we examined whether the stargazin-mediated increase in AMPAR surface expression as well as changes in the electrophysiological properties of the receptors are dependent on the amino acid at the Q/R site. We found that the extent and kinetics of receptor desensitization as well as the increase in kainate efficacy in the presence of stargazin are strongly dependent on the amino acid at the Q/R site. In contrast, the stargazin-mediated increases in surface expression appear to be entirely independent of the amino acid at the Q/R site, suggesting multiple functionally independent interactions between stargazin and AMPA receptors.

MATERIALS AND METHODS

Generation of Construct—Oligonucleotides were designed as degenerated mutagenesis PCR primers based on the sequence of GluR1(Q)flop (GenBank™ accession number X17184) to achieve saturating mutagenesis at the Q/R site of GluR1 in a single PCR, which was followed by subcloning. To check for successfully introduced mutations, a NarI restriction site was added as a silent mutation upstream of the codon for the Q/R site. Degenerated sense (5′-GGGCTTCTATTGTTTCT-3′) and antisense primers (5′-GTGAGATTTGACATTTC-3′), with D = (G/A/T), n = (A/C/G/T), H = (A/T/C)) were combined with antisense (5′-GGAGCCGGGATCCCGA-3′) and sense oligonucleotides (5′-CAGCTCCTAGTCTGGTCT-3′), respectively, following an “overlap extension” protocol. The resulting 729-bp PCR product was subcloned into GluR1(Q)flop/pSGEM using two BglII restriction sites flanking the mutated PCR-generated sequence. The randomly generated codon at the Q/R site of a large number of mutants was determined via sequencing to obtain all 19 combinations of the Q/R site. For expression in human embryonic kidney 293 (HEK293) cells, the flop isoforms of GluR1(Q), GluR1(S), and GluR1(T) were subcloned into pcDNA3 using the unique EcoRI and XhoI restriction sites. Stargazin was N-terminally tagged with enhanced cyan fluorescent protein (ECFP) by deletion of its restriction sites. Stargazin was N-terminally tagged with enhanced cyan fluorescent protein (ECFP) by deletion of its restriction sites. GluR1(Q), GluR1(S), and GluR1(T), and either ECFP-tagged stargazin or ECFP (3:2 ratio), using the calcium phosphate method. Transfection was performed for 16 h at 37 °C and 3% CO2. After transfection the medium was changed back to JMEM.

Labeling of Cell Surface Proteins Using Biotinylated ConA—Total membrane preparations and labeling of cell surface proteins with biotinylated concanavalin A were performed as described elsewhere (20, 21). In brief, 4–6 days after cRNA injection, 25 oocytes were incubated in 10 μM biotinylated concanavalin A (Sigma) in normal frog Ringer’s solution (NFR, containing in mM: 115 NaCl, 2.5 KCl, 1.8 CaCl2, 10 HEPES-NaOH, pH 7.2) at room temperature for 30 min. After three washes with NFR, intact oocytes were homogenized by several passages through a 100-μl pipette tip in ice-cold homogenization buffer (H-buffer: 20 μl/oocyte, containing 100 mM NaCl, 20 mM Tris-HCl, pH 7.4, 1% Triton X-100 plus a proteinase inhibitor mixture (Complete™ tablets, Roche Applied Science)). Homogenates were incubated at 4 °C on a rotator for 1 h. After centrifugation at 16,000 × g for 4 °C for 30 min, supernatants were carefully collected, supplemented with 50 μl of H-buffer-equilibrated streptavidin-agarose beads (Fluka, Taufkirchen, Germany), and incubated for 2 h at 4 °C on a rotator. Beads were precipitated, washed 5–6 times with ice-cold H-buffer, and finally boiled for 10 min in 50 μl of SDS-PAGE loading buffer (0.8 μl β-mercaptoethanol, 6% SDS, 20% glycerol, 25 mM Tris-HCl, pH 6.8, 0.1% bromphenol blue) to release the precipitated receptor protein.

Gel Electrophoresis and Western Blotting—Proteins were separated on 8% SDS-polyacrylamide gels (22) (Minirotein III, Bio-Rad) and transferred to nitrocellulose membranes (Hybond-ECL, Amersham Biosciences). Membranes were blocked for at least 2 h in 1× Rotiblock solution (Roth, Karlsruhe, Germany) supplemented with 140 mM NaCl and 20 mM Tris-HCl, pH 7.6, and probed overnight with affinity-purified rabbit antisera directed against the C termini of GluR1. The GluR1 antibody was a kind gift of Richard Huganir (Johns Hopkins University, Baltimore, MD). Peroxidase-coupled goat anti-rabbit IgG (Sigma) was used as the secondary antibody. All incubations were performed in 0.1× Rotiblock, 0.05% Tween 20, 140 mM NaCl, and 20 mM Tris-HCl, pH 7.6. Immunoreactive bands were visualized by the enhanced chemiluminescence method (SuperSignal West Pico, Pierce).

Electrophysiology—Two-electrode voltage clamp recordings from *Xenopus* oocytes were performed 4–6 days after injection of the cRNA at a holding potential of −70 mV using a TurboTec 10 CX amplifier (npi, Tamm, Germany). Currents were sampled at 50 Hz followed by low-pass filtering at 20 Hz. Data were digitized at 200 Hz using an ITCl6 computer interface (Instrutech Corp., Long Island, NY) as A/D converter and analyzed with Pulse 8.67 (Heka, Lambrecht, Germany). Recordings were performed in Ringer’s solution containing Mg2+-activated chloride channels (Mg-Ringer: 115 mM NaCl, 2.5 mM KCl, 1.8 mM MgCl2, and 8 mM CaCl2) at 20–22 °C. The duration of the test voltage pulse for desensitization was 100 ms at −40 mV. The clamping potential was +20 mV, and the holding potential was −60 mV. A current response for each desensitization pulse was recorded. Current responses for three consecutive pulses were averaged before analysis. Amplitude and time constant of the current responses were estimated using an analysis routine for single exponential decay (WinAnalyzer, Arts and Science Corp., Holliston, MA) based on a least-squares fitting routine.
Q/R Site Modulates Stargazin/AMPA Receptor Interaction

10 mM HEPES-NaOH, pH 7.2). Receptor agonists glutamate (300 μM) and kainate (150 μM) were prepared in Mg-Ringer and applied for 20 s. Cyclothiazide (100 μM) and naphthylspermine (10 μM) were prepared in Mg-Ringer containing glutamate (300 μM) or kainate (150 μM), respectively. Amplitudes were measured as steady-state currents after 20 s of agonist application. Electrodes were filled with 3 mM KCl and showed resistances of 0.3–1 megohms. cRNA was injected in at least two independent oocyte preparations always using the same cRNA preparation. cRNAs encoding for mutants that did not show robust currents even upon coexpression with stargazin were prepared again to rule out problems with RNA quality.

Whole-cell patch clamp recordings of HEK293 cells were performed 24–48 h after transfection at room temperature (21–24 °C) using an EPC-9 amplifier (Heka). Currents were digitized with a sampling rate of 10 kHz and low-pass filtered at 2.9 kHz. Pipettes were pulled from borosilicate glass and had resistances of 2–10 megohms. The extracellular solution contained 140 mM NaCl, 4 mM KCl, 2 mM CaCl2, 1 mM MgCl2, and 10 mM HEPES adjusted to pH 7.3 with NaOH. The pipette solution contained 130 mM CsF, 33 mM KOH, 4 mM NaCl, 2 mM MgCl2, 1 mM CaCl2, 11 mM EGTA, and 10 mM HEPES adjusted to pH 7.3 with KOH. Glutamate (1 mM) was prepared in an extracellular solution and applied for 200 ms. Rapid application of glutamate was performed using a two-channel θ glass capillary mounted on a piezoelectric translator. One channel contained continuously flowing extracellular solution, and the other contained the agonist. The patched cells were positioned in front of the channel containing the extracellular solution, and the digital/analog output of the computer was used to trigger the displacement and return of the piezoelectric translator moving the cell in front of the agonist-containing channel of the θ tube and back. Time constants of desensitization were calculated by single exponential fits with PulseFit 8.7 (Heka).

RESULTS

Stargazin Selectively Increases the Current Responses of Distinct Q/R Site Mutants of Glur1—Stargazin has been shown to modulate the trafficking and the electrophysiological properties of AMPA receptors (14, 17, 18, 23). As it is known that Q/R editing site of AMPA receptors is an important determinant of receptor complex assembly, trafficking, (7) and several electrophysiological properties (2–5), we examined whether and how different amino acids at the Q/R site influence the modulatory effects of stargazin on the AMPA receptor complex. Therefore, we mutated the Q/R site of Glur1 (“flop” isoform) to produce variants coding for each of the 20 standard amino acids and expressed these mutants in X. laevis oocytes. Responses to the application of glutamate and kainate were analyzed. All mutants showed agonist-evoked current responses much smaller (below 10 nA) than the wild type Glur1(Q), except for the mutant Glur1(N), which gave robust kainate-evoked responses (Fig. 1). These low currents could be because of low plasma membrane expression or specific electrophysiological properties of the mutants. Interestingly, the only mutant showing robust current responses for at least one of the agonists was the mutant that carried an asparagine, the amino acid that occurs in N-methyl-D-aspartate receptors at the position corresponding to the Q/R site.

To study the influence of the amino acid at the Q/R site on the interaction of the AMPAR with stargazin, we performed coexpression experiments with receptor mutants and stargazin. Upon coexpression of stargazin, with the exception of Glur1(V), Glur1(L), Glur1(M), Glur1(P), Glur1(H), Glur1(E), and Glur1(K), current responses were increased, either with both glutamate and kainate as the agonist or with only kainate (Glur1(A), Glur1(W), and Glur1(R); Fig. 1 and Table 1) as compared with Glur1(Q) in the absence of stargazin. The wild type Glur1(Q) and the mutants Glur1(N), Glur1(D), Glur1(C), and Glur1(Y) showed particularly strongly potentiated glutamate- and/or kainate-evoked current responses (potentiation factors >10) (Fig. 2; Table 1).

The large increases in current responses upon coexpression with stargazin could theoretically be caused by two distinctly different mechanisms. First, stargazin may selectively increase the trafficking to the plasma membrane of certain mutants; or second, stargazin could modulate the electrophysiological properties of the mutants. Both effects may depend on the amino acid at the Q/R site.

FIGURE 1. Stargazin (stg) strongly increases the current responses to application of 150 μM kainate (IKA) and 300 μM glutamate (IGLU) of Glur1(Q) as well as some of the Glur1 Q/R site mutants (two columns on the right). Typical current responses from Xenopus oocytes are shown for mutants with strongly increased current response to at least one of these agonists upon coexpression with stargazin (n > 9).
**TABLE 1**

**Normalized current responses of GluR1 wild type and GluR1 Q/R site mutants**

Normalized current responses and potentiation factors of GluR1 wild type and GluR1 Q/R site mutants were obtained in *Xenopus* oocytes in the presence and absence of stargazin (stg) (n = 8–41); recorded from 2–14 oocyte preparations. Current responses were normalized to wild type GluR1(Q) either in the presence or absence of stargazin; potentiation factors were normalized to GluR1(Q) in the absence of stargazin in every oocyte preparation.

| GluR1(X) | GluR1(X) | GluR1(X) + stg | Potentiation factors |
|---|---|---|---|
| | I_{Glu} | I_{KA} | I_{Glu} | I_{KA} | I_{Glu} | I_{KA} |
| Gln | 1.0 ± 0.0 | 1.0 ± 0.0 | 1.0 ± 0.0 | 1.0 ± 0.0 | 3.79 ± 0.99 | 17.78 ± 2.48 |
| Asn | 0.22 ± 0.11 | 0.86 ± 0.32 | 17.36 ± 2.87 | 2.41 ± 0.23 | 216.3 ± 55.6 | 103.7 ± 18.46 |
| Val | 0.08 ± 0.03 | 0.04 ± 0.01 | 0.01 ± 0.01 | 0.01 ± 0.00 | 0.18 ± 0.10 | 0.22 ± 0.04 |
| Leu | 0.31 ± 0.15 | 0.02 ± 0.01 | 0.03 ± 0.01 | 0.01 ± 0.00 | 0.14 ± 0.03 | 0.28 ± 0.08 |
| Ile | 0.06 ± 0.05 | 0.02 ± 0.01 | 0.25 ± 0.10 | 0.24 ± 0.07 | 2.32 ± 1.16 | 9.19 ± 3.88 |
| Ala | 0.04 ± 0.03 | 0.02 ± 0.01 | 0.08 ± 0.04 | 0.01 ± 0.01 | 0.75 ± 0.25 | 2.16 ± 0.58 |
| Met | 0.01 ± 0.01 | 0.02 ± 0.01 | 0.21 ± 0.13 | 0.02 ± 0.01 | 0.10 ± 0.03 | 0.31 ± 0.10 |
| Pro | 0.13 ± 0.06 | 0.20 ± 0.11 | 0.26 ± 0.09 | 0.01 ± 0.00 | 0.21 ± 0.09 | 0.04 ± 0.01 |
| Gly | 0.34 ± 0.08 | 0.07 ± 0.02 | 0.36 ± 0.09 | 0.20 ± 0.04 | 3.92 ± 1.47 | 8.43 ± 2.40 |
| Ser | 0.08 ± 0.04 | 0.03 ± 0.01 | 3.29 ± 0.63 | 1.64 ± 0.16 | 3.29 ± 0.63 | 1.64 ± 0.16 |
| Thr | 0.10 ± 0.04 | 0.03 ± 0.01 | 0.62 ± 0.18 | 0.43 ± 0.08 | 1.70 ± 0.60 | 8.07 ± 1.21 |
| Cys | 0.22 ± 0.08 | 0.22 ± 0.08 | 1.93 ± 0.41 | 1.82 ± 0.15 | 19.13 ± 7.57 | 90.77 ± 18.33 |
| His | 0.08 ± 0.02 | 0.03 ± 0.01 | 0.06 ± 0.02 | 0.03 ± 0.01 | 0.04 ± 0.02 | 0.25 ± 0.05 |
| Trp | 0.22 ± 0.08 | 0.01 ± 0.01 | 0.02 ± 0.01 | 0.08 ± 0.02 | 0.13 ± 0.07 | 3.06 ± 1.06 |
| Tyr | 0.15 ± 0.07 | 0.02 ± 0.00 | 3.99 ± 0.75 | 0.57 ± 0.07 | 16.17 ± 2.93 | 11.97 ± 1.47 |
| Phe | 0.07 ± 0.03 | 0.03 ± 0.01 | 0.80 ± 0.17 | 0.12 ± 0.04 | 1.24 ± 0.53 | 3.65 ± 1.71 |
| Glu | 0.32 ± 0.07 | 0.07 ± 0.03 | 0.20 ± 0.07 | 0.04 ± 0.01 | 0.10 ± 0.44 | 1.01 ± 0.40 |
| Asp | 0.22 ± 0.05 | 0.03 ± 0.01 | 1.44 ± 0.43 | 0.67 ± 0.06 | 13.22 ± 2.56 | 39.67 ± 3.74 |
| Arg | 0.06 ± 0.03 | 0.02 ± 0.003 | 0.30 ± 0.12 | 0.12 ± 0.02 | 1.14 ± 0.39 | 3.15 ± 0.41 |
| Lys | 0.15 ± 0.02 | 0.03 ± 0.01 | 0.04 ± 0.02 | 0.002 ± 0.001 | 0.11 ± 0.03 | 0.03 ± 0.003 |

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**FIGURE 2.** Stargazin strongly potentiates glutamate- and kainate-evoked current responses of several distinct Q/R site mutants of GluR1. Shown are potentiation factors of glutamate (A)- and kainate-evoked (B) currents in the presence of stargazin (current amplitude normalized to GluR1(Q) in the absence of stargazin in every oocyte preparation). *, p < 0.05; **, p < 0.01; ***, p < 0.001; significantly different from wild type GluR1(Q).

Stargazin Increases the Plasma Membrane Expression of the Receptor Mutants Only Slightly and in a Q/R Site-independent Manner—To examine whether the electrophysiological results obtained in the absence and presence of stargazin simply reflect the amount of receptor protein in the plasma membrane, we performed Western blot analyses of the plasma membrane expression of several representative mutants.

The coexpression of stargazin and GluR1(Q) led to an increase in plasma membrane expression of GluR1(Q) as described previously (14, 17, 18). In the absence of stargazin, the amounts of GluR1(Q), GluR1(R), and GluR1(S) protein were comparable, whereas GluR1(T) showed rather weak expression. By contrast, GluR1(N) was expressed even more strongly than GluR1(Q) (Fig. 3A). Thus, the small current responses of GluR1(R), GluR1(T), and GluR1(S) are not due to a reduced protein expression compared with GluR1(Q) and GluR1(N) but rather to differences in their intrinsic electrophysiological properties. Coexpression of stargazin did not change the intensities of the bands relative to each other (Fig. 3B).

A modest increase in the receptor amount in the plasma membrane upon coexpression of stargazin was detected for all mutants examined, similar to the wild type GluR1(Q). The fact that GluR1(Q) and GluR1(N) were expressed in the plasma membrane at higher levels than other mutants provides another hint that the homomeric assembly and trafficking to the plasma membrane of these two receptors may be favored. However, the moderate increase in protein amount upon stargazin coexpression cannot explain the huge increase in the current responses of these mutants. Therefore, we conclude that the observable increase in protein trafficking is not the main reason for the increase in current responses.

Stargazin Modulates the Extent of Desensitization and the Kainate Efficacy in a Manner Dependent on the Amino Acid at the Q/R Site—Next, we set out to test how the amino acid located at the Q/R site of the receptor mutants influenced the electrophysiological properties. The widely used ratio of kainate- to glutamate-evoked current amplitudes (KA/Glu ratio) is an indicator of two such properties, the kainate efficacy and the extent of desensitization (see e.g. Refs. 14 and 17). High KA/Glu ratios can be caused either by strong desensitization upon glutamate application, relative to kainate application, or by a high relative kainate efficacy. It has previously been shown that both of these properties are affected by the interaction of stargazin with AMPARs (14, 17, 18). The extent of desensitization is smaller upon coexpression with stargazin than for the receptor alone, whereas kainate efficacy is increased. Here, we found a wide range of KA/Glu ratios for the different mutants. Some, like GluR1(N) and GluR1(Y), showed relatively low KA/Glu ratios of 8.0 ± 4.3 and 7.8 ± 2.9, respectively, whereas other mutants like GluR1(S) and GluR1(T) showed KA/Glu ratios of 14.0 ± 1.9 and 26.8 ± 4.4, respectively, which are more similar...
to the wild type GluR1(Q) KA/Glu ratio of 43.2 ± 7.7 (Fig. 4, A and B). A low KA/Glu ratio indicates that either the desensitization of the receptor is relatively weak upon glutamate application or that the relative kainate efficacy is low. By contrast, a high KA/Glu ratio indicates strong glutamate-mediated desensitization or a high relative kainate efficacy. GluR1(W) showed an extremely high KA/Glu ratio of 296.2 ± 49.4, indicating an almost complete desensitization upon glutamate application or an extremely high kainate efficacy. To distinguish whether the mutants showing a higher KA/Glu ratio than wild type GluR1(Q) are stronger desensitizing, or have higher kainate efficacies, we determined the KA/Glu ratio while reducing glutamate-induced desensitization by coapplication of cyclothiazide (CTZ) and the saturating agonist concentrations. The concentrations used were definitely saturating, even in the presence of CTZ, because CTZ as well as stargazin shifts the EC50 to lower values (14, 17, 24). In the presence of CTZ during glutamate application, all KA/Glu ratios decreased to values between 1.3 ± 0.08 for GluR1(N) and 4.5 ± 0.68 for GluR1(W) (Fig. 4, A and C), indicating that the main effect of stargazin on the receptor mutants is a modulation of the extent of desensitization, which is strongly dependent on the amino acid at the Q/R site. The remaining differences in the KA/Glu ratios may have been because of a Q/R site-dependent modulation of the kainate efficacy or could have been caused by a reportedly incomplete (24) CTZ-mediated abolishment of desensitization in flop isoforms. GluR1-flop was used in this study because it is the splice variant presumably expressed in the adult brain (25). Evidence that kainate efficacy is also affected by the interaction of stargazin with the receptor has been reported previously (17).

To test whether the remaining differences in the KA/Glu ratios found in the presence of CTZ were due to incomplete inhibition of receptor desensitization by CTZ or reflected a differentially modulated kainate efficacy, we combined several Q/R site mutants carrying in addition the L479Y mutation, determined in the presence of stargazin (Fig. 4D). The observed KA/Glu ratios were smaller than 1, indicating that glutamate is the agonist with the higher efficacy of receptor gating, despite the fact that the efficacy of kainate is increased tremendously by the coexpression of stargazin.
In the absence of desensitization, a differently modulated stargazin-mediated increase in kainate efficacy became discernible. GluR1(T) and GluR1(S) showed the strongest and GluR1(W) the weakest increase in kainate efficacy, and the kainate efficacy of all other tested mutants was increased to roughly the same extent. This differently modulated kainate efficacy was masked by the incomplete inhibition of desensitization in the experiments performed with CTZ. During coapplication of glutamate and CTZ, GluR1(W) showed the highest KA/Glu ratio, which, taken at face value, might be interpreted as indication of the strongest increase in kainate efficacy among all mutants tested. However, complete inhibition of desensitization through the L479Y mutation surprisingly revealed the lowest KA/Glu ratio for GluR1(W) compared with all mutants tested, demonstrating a rather weak increase in kainate efficacy. We conclude from those data that kainate efficacy, as well as the extent of desensitization and the desensitization kinetics are modulated by stargazin in a manner dependent on the amino acid at the Q/R site (27). Thus, an interaction of stargazin with the cellular spermines in a voltage-dependent manner (27). These spermines are believed to interact directly with the amino acid at the Q/R site (27). These spermines are believed to interact directly with the amino acid at the Q/R site (27). These spermines are believed to interact directly with the amino acid at the Q/R site (27). These spermines are believed to interact directly with the amino acid at the Q/R site (27). These spermines are believed to interact directly with the amino acid at the Q/R site (27). These spermines are believed to interact directly with the amino acid at the Q/R site (27).

Stargazin Modulates the Kinetics of Receptor Desensitization in a Manner Dependent on the Amino Acid at the Q/R Site—It has been shown recently that the desensitization kinetics of AMPARs are affected by stargazin (14, 18). Given that we found that stargazin strongly modulates the extent of desensitization depending on the amino acid at the Q/R site, we next examined whether the kinetics of desensitization are also affected. Therefore, we coexpressed the mutants GluR1(S) and GluR1(T) as well as the wild type GluR1(Q) with stargazin in HEK293 cells and compared their desensitization time constants (τ) with the non-desensitizing component for GluR1(Q) by 2-fold, from 0.8 ± 0.3% without stargazin to 1.7 ± 0.5% when coexpressed with stargazin. In the presence of stargazin, the mutants show non-desensitizing components of 2.3 ± 0.8% (GluR1(T)) and 3.7 ± 1.1% (GluR1(S)) compared with the completely desensitizing current responses obtained in the absence of stargazin. This is additional proof that the extent of desensitization and the desensitization kinetics are modulated by stargazin in a manner dependent on the amino acid at the Q/R site.

Stargazin Interacts with the Q/R Site via an Allosteric Effect—To test whether the Q/R site-dependent modulatory effects of stargazin are due to an allosteric effect or whether stargazin interacts directly with the pore region of the receptor complex, we measured the block of the open channel by naphtylspermine (NASP) in the presence and absence of stargazin. AMPA receptors with a glutamate at the Q/R site are blockable by extracellular spermines in a voltage-dependent manner (27). These spermines are believed to interact directly with the amino acid at the Q/R site (27). Thus, an interaction of stargazin with the amino acid at the Q/R site or in its vicinity would probably interfere with the NASP-mediated block of the open channel. Kainate-evoked currents of GluR1(Q), GluR1(N), and GluR1(C), the only three receptor variants that showed reliably analyzable currents in the absence of stargazin, failed to show a decrease in block by extracellular NASP in the presence of star-
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FIGURE 6. Stargazin (stg) does not alter the block of the open channel by NASP. 10 μM NASP is coapplied (intra- or extracellularly) with 150 μM kainate (n = 4–13). p > 0.05, compared with GluR(X) in the absence of stargazin.

to glutamate- or kainate-evoked current responses that are smaller than wild type and generally below 10 nA except for GluR1(N), which showed robust kainate-evoked currents. Because our Western blot analyses did not reveal differences in the amount of receptor protein in the plasma membrane that were sufficient to explain the huge differences in current responses, we conclude that the small current responses obtained for most mutants were due to altered electrophysiological properties such as fast desensitization or low single channel conductance. Although the protein amount in the plasma membrane for GluR1(N) is only slightly larger than that for GluR1(Q), GluR1(R), and GluR1(S), homomeric receptor complexes consisting of this particular receptor subunit mutant may be exported from the ER preferentially, probably because of favorable receptor assembly in the ER as reported for GluR2(Q) (7). Differences in relative plasma membrane expression compared with the results of Greger et al. (7), especially for GluR1(R), may be due to the different expression systems (oocytes versus primary hippocampal neurons) and methods of analysis (Western blot versus pulse-chase analysis).

Coexpression of stargazin led to increased current responses upon glutamate and kainate application for most mutants, although the observed increase varied markedly among the mutants. Strongly increased current responses were obtained for very diverse mutants that have amino acids at their Q/R sites that differ significantly in shape and volume as well as in their physicochemical properties such as charge or polarity (Gln, Asn, Asp, Cys, Tyr). Thus, there appears to be no obvious correlation between the chemical nature of the amino acid at the Q/R site and the extent of the stargazin-mediated increase in current response.

It has already been shown that both stargazin and the amino acid at the Q/R site of AMPARs, independently of each other, are regulators of the trafficking of AMPA receptor complexes from the ER to the plasma membrane and that both are important determinants for several electrophysiological properties of the receptors ion channel (2–5, 7, 14, 17, 18). Building on these findings, we set out to analyze whether there is any correlation between the amino acid at the Q/R site and the effect of stargazin on surface expression and electrophysiological properties of GluR1 and, therefore, interplay between the two modulators. We also examined the influence of the amino acid at the Q/R site of the AMPAR GluR1 on the interaction between the receptor and stargazin.

We constructed mutants of this receptor that had the genomically encoded glutamine at the narrow constriction of the ion channel exchanged individually against all other 19 canonical amino acids. We show that, in the absence of stargazin, expression of these mutants in Xenopus laevis oocytes leads
nation of Q/R site mutants with the L479Y mutation, which is known to virtually abolish receptor desensitization (26), revealed that the stargazin-mediated increase in kainate efficacy is also dependent on the amino acid at the Q/R site.

An increase in kainate efficacy as found for all Q/R site mutants examined in our study has been reported previously for GluR1(Q) (17). It is probably because of an increased closure of the clam shell-shaped binding cleft, a conformational change that has been shown to cause higher current responses and slightly increased receptor desensitization (30).

The Q/R site-dependent reduction of the extent of desensitization by stargazin seen in *Xenopus* oocytes is confirmed by results obtained in our patch clamp recordings from HEK293 cells. Additionally, we found a prominent stargazin-mediated slowing of the desensitization kinetics of the receptors. This slowing was also dependent on the amino acid at the Q/R site. Such an effect on desensitization kinetics has been reported previously for the wild types of GluR1(Q) and GluR4(Q), as well as for GluR2(Q) (14, 17, 18).

As expected, stargazin did not change the block of the open channel by either intra- or extracellular application of NASP, indicating an allosteric mechanism of Q/R site-mediated modulatory effects rather than a direct interaction of this site with stargazin.

In summary, we have shown for the first time that the main effect of stargazin on GluR1, the modulation of the extent of desensitization, is Q/R site-dependent. This finding is of physiological relevance because most AMPAR complexes occurring in cells. Additionally, we found a prominent stargazin-mediated slowing of the desensitization kinetics of the receptors. This slowing was also dependent on the amino acid at the Q/R site. Such an effect on desensitization kinetics has been reported previously for the wild types of GluR1(Q) and GluR4(Q), as well as for GluR2(Q) (14, 17, 18).

As expected, stargazin did not change the block of the open channel by either intra- or extracellular application of NASP, indicating an allosteric mechanism of Q/R site-mediated modulatory effects rather than a direct interaction of this site with stargazin.

In summary, we have shown for the first time that the main effect of stargazin on GluR1, the modulation of the extent of desensitization, is Q/R site-dependent. This finding is of physiological relevance because most AMPAR complexes occurring naturally are composed of subunits that have two different amino acids at their Q/R sites, for example GluR1(Q) or GluR3(Q) complexed with GluR2(R) (31).

How can our observations be explained on a structural basis? Given that we showed in this study that stargazin influences receptor desensitization, it is likely that stargazin interacts with receptor sites that are involved in the regulation of desensitization. To our knowledge only two regions in the receptor have been positively identified thus far as participating in the desensitization of the receptor. These regions are the ligand-binding domain and the linker region between the second transmembrane domain and the S2 domain (26, 32–34). Crystallization of the ligand-binding domain of GluR2 has revealed that it forms dimers in which two monomers are positioned “back-to-back” (30, 35), whereas the contact between the dimers is made by their L1 lobes, which consist of parts of the S1 and S2 loops, thereby forming the upper part of the ligand-binding domains. All residues known to influence desensitization are localized at this dimer-dimer interface (36). A recent study revealed, furthermore, that mutation of distinct residues involved in the dimer-dimer interaction leads to a strong increase in desensitization of the mutants (37). By contrast, a decrease in receptor desensitization can be caused by interaction with stargazin (14, 17, 18). This effect appears to be mediated by the first extracellular loop of stargazin (14, 23).

We observed that the amount of protein inserted in the plasma membrane apparently was not strongly affected. Therefore, we propose that the Q/R site allosterically alters the structure of the lipid-exposed side of the transmembrane domains, to which stargazin is likely to bind (38, 39). This alteration in turn changes the interaction between stargazin and the receptor at additional sites of contact, such as the first extracellular loop of stargazin, and thereby alters the desensitization and other functional properties of the receptor. These findings are surprising, because a small change in the pore region of the receptor, which is probably not even directly involved in the interaction with stargazin, has a strong impact on the properties of the receptor-stargazin complex and likely alters its physiological function.

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