Stargazin controls the pharmacology of AMPA receptor potentiators

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Contributed by Roger A. Nicoll, April 19, 2006

Glutamate is the major excitatory neurotransmitter in brain, and α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-type glutamate receptors (AMPARs) mediate the majority of postsynaptic depolarization. AMPAR ion channels display rapid gating, and their deactivation and desensitization determine the timing of synaptic transmission. AMPAR potentiators slow channel deactivation and desensitization, and these compounds represent exciting therapies for mental and neurodegenerative diseases. Previous studies showed that the AMPAR potentiators cyclothiazide and 4-[2-(phenylsulfonylamino)ethylthio]-2,6-difluorophenoxyacetamide display a preference for flip and flop alternatively spliced versions of glutamate receptor subunits, respectively. Here, we find that the AMPAR auxiliary subunit stargazin changes this pharmacology and makes both spliced forms of glutamate receptor subunit 1 sensitive to both classes of potentiator. Stargazin also enhances the effect of AMPAR potentiators on channel deactivation. This work demonstrates that stargazin controls AMPAR potentiator pharmacology, which has important implications for development of AMPAR potentiators as therapeutic agents.

transmembrane AMPA receptor regulatory protein | (TARP) | glutamate | synapse | psychiatry | cognition

Most excitatory transmission in brain occurs at synapses that use glutamate as the neurotransmitter. The α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-type receptors (AMPARs) are glutamate-gated cation channels. Activation of these receptors provides most of the postsynaptic depolarization that induces neuronal firing. AMPAR channels show complex gating kinetics and vary considerably, depending on the subunit composition of the AMPAR (1). These channels open rapidly upon glutamate binding. The channels then desensitize quickly; that is, despite continued binding to agonist, the channels close with a desensitization time constant, τ_{des} , of 1–15 ms. At most brain synapses, glutamate diffuses out of the cleft rapidly, even faster than AMPARs desensitize. The unbinding of glutamate from the receptor closes the channel, a process known as deactivation ($\tau_{\text{dea}} \approx 1$ –3 ms). The timing of synaptic glutamate release and synaptic glutamate clearance interplays with AMPAR desensitization and deactivation. Collectively, these processes shape excitatory postsynaptic currents.

AMPARs are heterotetramers composed of glutamate receptor subunits 1–4 (GluR1–4) (2, 3). Each subunit can be alternatively spliced as either a “flip” (e.g., GluR1i) or a “flop” (e.g., GluR1o) version (4). This alternative splicing regulates channel kinetics, because the flop version desensitizes and deactivates more rapidly than does the flip version. In addition to the GluR pore-forming subunits, neuronal AMPARs also contain stargazin-like auxiliary subunits known as “transmembrane AMPAR regulatory proteins” (TARPs) (5). TARPs mediate AMPAR surface expression and synaptic clustering (5) and also modulate AMPAR channel gating by slowing desensitization and deactivation (6, 7).

AMPA potentiators are an exciting class of experimental therapeutics that promote AMPAR signaling by blunting desensitization and slowing deactivation (8). By promoting excitatory transmission, these agents show robust activity in a variety of

preclinical models. AMPAR potentiators enhance cognition, ameliorate depression, and lessen neurodegeneration in several animal models (8–13). In addition, AMPAR potentiators show discrete subunit specificity. The prototypical AMPAR potentiator, cyclothiazide, selectively potentiates responses of flip-type GluR subunits (14). On the other hand, the potentiator 4-[2-(phenylsulfonylamino)ethylthio]-2,6-difluorophenoxyacetamide (PEPA) acts specifically on flop isoforms (15). Here, we asked whether the stargazin subunit of AMPAR modulates the pharmacology of these potentiator drugs.

Results

We used the *Xenopus laevis* oocyte expression system to evaluate stargazin effects on AMPAR function. As reported previously (6, 16), coinjection of stargazin cRNA increases glutamate-evoked currents from oocytes injected with GluR1i. To best compare the properties of channels containing GluR1 alone vs. GluR1 plus stargazin, we sought to study oocytes that had similar amounts of surface GluR1. We modulated surface receptor numbers by injecting oocytes with different amounts of stargazin and hemagglutinin epitope (HA)-GluR1i cRNA and quantified surface GluR1 by chemiluminescence as described in ref. 6. We found similar numbers of GluR1 surface receptors from oocytes injected with 0.5 ng of GluR1i alone and from those injected with 0.1 ng of GluR1i plus 0.1 ng of stargazin (Fig. 1A). As reported previously (6, 16), cyclothiazide greatly increased glutamate-evoked currents, both in oocytes injected with GluR1i alone and in those injected with GluR1i plus stargazin (Fig. 1A). Consistent with previous studies (14), we found that cyclothiazide has only modest effects on GluR1o expressed in oocytes (Fig. 1B). However, cyclothiazide greatly potentiated glutamate-evoked currents from oocytes expressing GluR1o plus stargazin (Fig. 1B). In the presence of stargazin, cyclothiazide increased steady-state currents in GluR1o-expressing oocytes by ≈ 15 -fold (Fig. 1B).

We performed analogous experiments exploring potentiation with PEPA, an AMPAR potentiator that preferentially affects flop receptors (15). As shown previously, we found that PEPA had minimal effects on GluR1i (Fig. 1C). In contrast, PEPA greatly increased currents from oocytes expressing GluR1i plus stargazin (Fig. 1C). As expected, GluR1o channels showed significant potentiation with PEPA; this potentiation was further increased by coinjection of GluR1o with stargazin.

To explore the effect of stargazin and AMPAR potentiators on channel desensitization and deactivation, we used a rapid perfusion

Conflict of interest statement: No conflicts declared.

Abbreviations: AMPA, α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; AMPAR, AMPA receptor; GluR, glutamate receptor subunit; PEPA, 4-[2-(phenylsulfonylamino)ethylthio]-2,6-difluorophenoxyacetamide; TARP, transmembrane AMPAR regulatory protein; HA, hemagglutinin epitope.

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Fig. 3. Stargazin (STG) enhances GluR1 affinity for cyclothiazide. Steady-state currents evoked by glutamate (10 μ M) were recorded in oocytes injected with GluR1i or GluR1o in the presence or absence of stargazin. Results are presented normalized to current with 100 μ M cyclothiazide. For both GluR1i and GluR1o, stargazin shifted the EC_{50} for cyclothiazide to the left (EC_{50} for GluR1i = 10 μ M, GluR1i/stargazin = 2 μ M, GluR1o = 29 μ M, GluR1o/stargazin = 10 μ M).

Discussion

This study demonstrates that stargazin modulates the pharmacology of AMPAR potentiators. Stargazin shows additive effects with AMPAR potentiators to blunt the extent of desensitization and to slow deactivation. Furthermore, stargazin increases the affinity of AMPAR potentiators for glutamate receptor subunits. Interestingly, stargazin modulates the subunit specificity of AMPAR potentiators to make flop receptors sensitive to cyclothiazide and flip receptors sensitive to PEPA. These data provide insight into the mechanisms by which stargazin and AMPAR potentiators modulate channel activity and have implications for the development of AMPAR potentiators in the treatment of neurological diseases.

The mechanism for stargazin modulation of AMPAR kinetics is not certain. Molecular chimera analyses showed that the first extracellular loop of stargazin is essential for controlling AMPAR channel properties (6). Similarly, cyclothiazide influences channel function by interacting near the glutamate-binding pocket in the extracellular domain of the receptor (18). Because stargazin modulates the interaction and effects of AMPAR potentiators, it seems likely that the extracellular loop of stargazin may also interact near the glutamate-binding site. This model would be consistent with the increase in glutamate affinity caused by stargazin (6, 7).

Previous studies have defined at least two classes of AMPAR potentiators. Whereas two cyclothiazide molecules bind in the GluR subunit dimer interface to block desensitization (18), a single aniracetam molecule binds at the center of the dimer interface to slow channel deactivation and desensitization of AMPARs (19, 20). Future structure studies of stargazin with these different types of AMPAR potentiators should provide valuable insights.

Cyclothiazide and stargazin affect desensitization and deactivation of AMPARs independently and additively. Because cyclothiazide blocks desensitization of AMPAR flip isoforms alone and with stargazin, these results suggest that stargazin acts on the opening of AMPARs but may not modulate entry into and out of desensitized states. In support of this model, single-channel analysis revealed that stargazin modulates the gating of AMPARs (6).

The modulation of AMPAR potentiators specificity for alternatively spliced channel subunits is striking. Numerous previously published studies have established that many AMPAR potentiators affect only one of the two alternatively spliced versions of transfected AMPAR subunits. The prototypical AMPAR potentiator, cyclothiazide, specifically augments re-

sponses from flip-type channels (14). This differential effect of cyclothiazide has been used extensively to define the relative expression of flip vs. flop isoforms in neuronal populations (21, 22). Our results showing that stargazin modulates the subunit specificity of AMPAR potentiators to make flop receptors sensitive to cyclothiazide add complexity to these analyses.

This study has focused on stargazin; however, there are three additional TARPs that modulate the biophysical properties of AMPAR channels (6, 23). TARPs are differentially expressed in discrete neuronal populations throughout the brain. Notably, stargazin is enriched in cerebellum, γ -3 in cerebral cortex, γ -4 in developing brain, and γ -8 in hippocampus (23). Whether TARP isoforms differentially modulate the pharmacology of AMPARs, and whether this specificity can explain the differential responses found in various neuronal populations, will require further study.

This work has implications for the clinical pharmacology of AMPAR potentiators. Preclinical and early clinical studies have shown that AMPAR potentiators can enhance cognitive function as nootropic agents and have therapeutic potential in a variety of mental and neurodegenerative diseases, including schizophrenia, depression, and Parkinson's disease (8–11). Discovery of the TARP family of AMPAR auxiliary subunits should facilitate development of clinically useful AMPAR potentiators.

Materials and Methods

Electrophysiology Using *X. laevis* Oocytes. Two-electrode voltage-clamp recordings were performed as described in ref. 24. Briefly, GluR1i, GluR1o, and stargazin were subcloned into pGEM-HE vector. cRNAs were transcribed *in vitro* by using T7 mMessage mMachine (Ambion, Austin, TX) and injected into oocytes. Two days after injection, levels of cell surface HA-GluR1 were quantitated by chemiluminescence as described in ref. 6. Oocytes expressing similar amounts of receptor were subjected to two-electrode voltage-clamp analysis (redox holding potential, $E_h = -70$ mV), which was performed at room temperature in recording solution containing 90 mM NaCl, 1.0 mM KCl, 1.5 mM $CaCl_2$, and 10 mM Hepes (pH 7.4).

Outside-Out Patch Recordings. Outside-out patches from injected oocytes were obtained as described previously (25). Outside-out patch recording was carried out with an EPC-8 amplifier (HEKA Electronics, Lambrecht/Pfalz, Germany) under continuous perfusion with frog Ringer's solution (115 mM NaCl/2 mM KCl/2 mM $CaCl_2$ /10 mM Hepes, adjusted to pH 7.2 with NaOH). The patch pipette was prepared from borosilicate glass capillaries (WPI Instruments, Waltham, MA) and had 4- to 7-M Ω input resistance when filled with 100 mM KCl/2 mM $MgCl_2$ /10 mM EGTA/10 mM Hepes, adjusted to pH 7.2 with KOH. Responses were filtered at 10 kHz and digitized at 26 μ s per point. The holding potential was at -60 mV. Fast application of glutamate was performed by using the methods described previously (25). Briefly, glutamate (10 mM) was applied by perfusion of the patch membrane with θ tubes driven by a piezo manipulator (PZ-150M; Burleigh Instruments, Fishers, NY). After recording, the patch membrane was blown off and the junction current between the control solution and 10% frog Ringer's solution was measured to monitor solution exchange without moving the patch pipette and θ tube. Responses to glutamate having a 20–80% rise time <400 μ s were used for analysis. The decay phase of the response was fitted with single-exponential functions by using IGOR PRO (WaveMetrics, Lake Oswego, OR).

We thank James R. Howe (Yale Medical School, New Haven, CT) for insightful discussions. This work was supported by grants from the National Institutes of Health (to D.S.B. and R.A.N.). K.W. is supported by grants-in-aid for Scientific Research from the Ministry of Health, Labor and Welfare of Japan and from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

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