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COMPARATIVE ANALYSIS OF THE PHARMACOLOGY OF GLUR1 IN COMPLEX WITH TRANSMEMBRANE AMPA RECEPTOR REGULATORY PROTEINS γ2, γ3, γ4, AND γ8

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Abstract—AMPA receptors (AMPARs) mediate the majority of fast synaptic transmission in the CNS of vertebrates. They are believed to be associated with members of the transmembrane AMPA receptor regulatory protein (TARP) family. TARPs mediate the delivery of AMPA receptors to the plasma membrane and mediate their synaptic trafficking. Moreover, TARPs modulate essential electrophysiological properties of AMPA receptors. Here, we compare the influence of rat TARPs (γ2, γ3, γ4, and γ8) on pharmacological properties of rat GluR1(Q)flip. We show that agonist potencies are increased by all TARPs, but to individually different extents. On the other hand, all TARPs increase agonist potencies at the virtually non-desensitizing mutant GluR1-L479Y almost identically. Comparison of the influence of individual TARPs on relative agonist efficacies confirmed that the TARPs can be functionally subdivided into two subgroups, one consisting of γ2 and γ3 and one consisting of γ4 and γ8. Surprisingly, we found that TARPs convert certain AMPA receptor antagonists to agonists. The potency of one of these converted antagonists is dependent on the particular TARP. Moreover, TARPs (except γ4) reduce the ion channel block by the synthetic Joro spider toxin analog 1-naphthylacetyl spermine (NASP). In addition, TARPs increase the permeability of the receptor to calcium, indicating that TARPs directly modulate important ion pore properties. In summary, the data presented herein will illustrate and help to understand the previously unexpected complexities of modulation of AMPA receptor pharmacological properties by TARPs.

Key words: *stargazin*, AMPA receptor, glutamate receptor, NASP, CNQX, quinoxaline.

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Abbreviations: CaR, Ca-Ringer; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; CTD, C-terminal domain; CTZ, cyclothiazide; DNQX, 6, 7-dinitroquinoxaline-2,3(1H,4H)-dione; EGTA, ethylene glycol-bis(2-aminoethyl ether)-N,N,N′,N′-tetraacetic acid; E_{rev}, reversal potential; Glu, L-glutamate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IC_{50}, inhibitory concentration; I–V, current–voltage relationship; KA, kainate; LBD, ligand binding domain; MgR, magnesium frog Ringer's solution; NASP, 1-naphthylacetyl spermine; NBQX, 1,2,3,4-tetrahydro-6-nitro-2,3-dioxo-benzo[f]quinoxaline-7-sulfonamide; S.E.M., standard error of the mean; TARP, transmembrane AMPA receptor regulatory protein; TCM, trichlormethiazide.

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AMPA receptors mediate most of the fast excitatory synaptic transmission in the CNS of vertebrates. This subfamily of the ionotropic glutamate receptors (iGluRs) consists of four subunits, GluR1–GluR4 (Hollmann and Heinemann, 1994), forming functional homo- and heterotetrameric receptor complexes (Rosenmund et al., 1998; Hollmann, 1999). Additional diversity in the family of AMPA receptors is achieved by multiple posttranscriptional modifications. Alternative splicing leads to two major variants of each AMPA receptor subunit, named flip and flop (Sommer et al., 1990). Further, RNA editing at the so-called Q/R site of GluR2 determines surface expression of the GluR2-containing receptor complexes (Greger et al., 2002, 2003) and their electrophysiological properties such as the conductance for divalent cations (Hollmann et al., 1991; Hume et al., 1991; Swanson et al., 1997).

In vivo, AMPA receptors most likely are always heteromers, assembled in neurons as either GluR1/GluR2 or GluR2/GluR3 complexes (Wenthold et al., 1996), which are virtually impermeable to calcium ions (Burnashev et al., 1995). In addition, some populations of glial cells express calcium-permeable GluR1/GluR4 receptor complexes (Ripellino et al., 1998). Functional AMPA receptors *in vivo* appear to be associated with members of the family of transmembrane AMPA receptor regulatory proteins, or TARPs (Vandenberghe and Bredt, 2005; Nakagawa et al., 2005). The family of TARPs is believed to consist of five members: γ2, γ3, γ4, γ7, and γ8 (Tomita et al., 2003; Kato et al., 2007). Each member mediates synaptic transport and localization of AMPA receptors. This mechanism is the key to functional synaptic AMPA receptors, as TARP-lacking neurons do not display AMPA receptor-mediated synaptic transmission (Hashimoto et al., 1999; Chen et al., 2000; Rouach et al., 2005). In the last 3 years, several publications demonstrated that, in addition to their effects on AMPA receptor transport, TARPs have a strong influence on key electrophysiological properties of AMPA receptors both *in vivo* and in heterologous expression systems (Yamazaki et al., 2004; Priel et al., 2005; Tomita et al., 2005; Turetsky et al., 2005). The extent of modulation of the electrophysiological properties of AMPA receptors is dependent on the associated TARP (Kott et al., 2007; Körber et al., 2007b; Milstein et al., 2007; Cho et al., 2007). Furthermore, the AMPA receptor subunit itself determines the extent of TARP-mediated modulation (Kott et al., 2007; Körber et al., 2007b; Turetsky et al., 2005).

As TARPs modulate several electrophysiological properties of AMPA receptors, it is highly likely that association with a TARP will also alter pharmacological properties of

AMPA receptors. First evidence of TARP-induced modulation of AMPA receptor pharmacology was provided by the much stronger potentiation of responses induced by kainate (KA) compared with those induced by glutamate (Glu) at certain AMPA receptors (Kott et al., 2007). Nearly all TARPs increase the potency of the full agonist glutamate at GluR1 receptors, and the extent of the increase in glutamate potency is TARP-dependent (Kott et al., 2007; Yamazaki et al., 2004). However, γ7 is an exception as it does not increase glutamate potency at GluR1 (Kato et al., 2007). In addition, the potency of the AMPA receptor partial agonist KA is also increased by *stargazin* (γ2) (Turetsky et al., 2005). A new study indicates that modulation of KA potency of AMPA receptors, similar to glutamate potency, may be dependent on the coexpressed TARP (Milstein et al., 2007). Furthermore, TARPs lower the efficacy of desensitization inhibitors such as cyclothiazide (CTZ) or trichlormethiazide (TCM) (Kott et al., 2007). A recent publication showed that *stargazin* attenuates the block of the ion pore of AMPA receptors by polyamines (Soto et al., 2007).

In this study we analyzed the influence of the four TARPs predominantly expressed in the CNS (γ2, γ3, γ4, and γ8) on the pharmacology of the AMPA receptor GluR1(Q)flip. We focused our investigation on several key pharmacological properties such as relative agonist efficacy, agonist potencies, and efficacy of antagonists. Furthermore, we analyzed TARP-dependent modulation of AMPA receptor ion pore block by the synthetic Joro spider toxin analog 1-naphthylacetyl spermine (NASP). Experiments were performed in *Xenopus laevis* oocytes, which allow a detailed characterization of AMPA receptor pharmacology in the presence and absence of TARPs without interference of endogenous subunits.

EXPERIMENTAL PROCEDURES

cRNA synthesis

The cDNA of the flip-isoformed rat GluR1(Q) receptor subunit as well as the cDNAs of the four rat TARPs were subcloned in the expression vector pSGEM that is optimized for expression in *Xenopus laevis* oocytes (Villmann et al., 1997). cRNA synthesis was performed as described earlier (Villmann et al., 1999). Briefly, template DNA was linearized with a suitable restriction enzyme. cRNA was synthesized from 1 μg of linearized DNA using an *in vitro* transcription kit (Fermentas, St. Leon-Rot, Germany) and a modified protocol that employs 400 μM GpppG (GE Healthcare, Freiburg, Germany) for capping and an extended reaction time of 3 h with T7 polymerase. Trace labeling was performed with α-[^{32}P]UTP to allow calculation of yields and evaluation of transcript quality by formaldehyde agarose gel electrophoresis.

Preparation of *Xenopus laevis* oocytes

Frog oocytes of stages V or VI were surgically removed from the ovaries of *Xenopus laevis* (Nasco, Fort Atkinson, WI, USA) anesthetized with 3-aminobenzoic acid ethylester (1.5 g/l; Sigma-Aldrich, Taufkirchen, Germany). All experiments conformed to German and international guidelines on the ethical use of animals. Every effort was made to minimize the number of animals used and their suffering. Lumps of ~20 oocytes were incubated for 1.5 h with 784 U/ml (4 mg/ml) collagenase type I (Worthington, Lakewood, NJ, USA) in Ca^{2+}-free Barth's solution (88 mM NaCl, 1.1 mM KCl, 2.4 mM $NaHCO_3$, 0.8 mM $MgSO_4$, 15 mM Hepes-NaOH pH 7.6) with slow agitation to remove the follicular cell layer, then washed extensively with Barth's solution (88 mM NaCl, 1.1 mM KCl, 2.4 mM $NaHCO_3$, 0.3 mM $Ca(NO_3)_2$, 0.4 mM $CaCl_2$, 0.8 mM $MgSO_4$, 15 mM Hepes-NaOH pH 7.6). Oocytes were maintained in Barth's solution supplemented with 100 μg/ml gentamicin, 40 μg/ml streptomycin, and 63 μg/ml penicillin. Defolliculated oocytes were injected with 2 ng of cRNA when GluR1(Q)flip was expressed alone and with 2.2 ng for coexpression experiments with TARPs (2 ng GluR1(Q)flip cRNA and 0.2 ng TARP cRNA) using a nanoliter injector (WPI, Sarasota, FL, USA). In case of expression of heteromeric AMPA receptors, both cRNAs were mixed before injection, and 2 ng of the mixture was injected per oocyte.

Standard electrophysiological measurements in *Xenopus laevis* oocytes

Four to five days after injection, oocyte current responses were recorded in magnesium frog Ringer's solution (MgR; 115 mM NaCl, 2.5 mM KCl, 1.8 mM $MgCl_2$, 10 mM Hepes-NaOH pH 7.2) under voltage clamp at −70 mV holding potential with a TurboTec 10CX amplifier (npi, Tamm, Germany) controlled by Pulse 7.53 software (HEKA, Lambrecht, Germany). Recording pipettes were pulled from borosilicate glass (Hilgenberg, Malsfeld, Germany) using a PIP5 pipette vertical puller (HEKA). Voltage electrodes had resistances of 1–4 MΩ and were filled with 3 M KCl; current electrodes had resistances of 0.5–1.5 MΩ and were filled with 3 M CsCl. Compounds were applied for 20 s by superfusion at a flow rate of ~5 ml/min. Glutamatergic agonists were prepared in MgR at the following saturating concentrations: 300 μM L-glutamate (Glu), 150 μM, 10 μM AMPA, and 10 μM domoate (DOM). AMPA receptor antagonists were also prepared in MgR, and each tested compound had a concentration of 10 μM. Tested compounds were 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), 6,7-dinitroquinoxaline-2,3(1H,4H)-dione (DNQX), and 1,2,3,4-tetrahydro-6-nitro-2,3-dioxo-benzo[f]quinoxaline-7-sulfonamide (NBQX). To block receptor desensitization 300 μM glutamate or 10 μM CNQX was each supplemented with 600 μM TCM. Steady-state current responses of one series of measurements were averaged, and the standard error of the mean (S.E.M.) was calculated. Statistical significance was determined with an unpaired, two-sided Student's *t*-test.

Determination of agonist/antagonist potencies

To determine EC_{50} or inhibitory concentration (IC_{50}) values for the tested compounds, 10–12 different concentrations were applied in increasing concentrations to the same oocyte, and steady-state amplitudes of the evoked currents were measured. The obtained steady-state responses were normalized to the maximal detected amplitude induced by saturating concentrations of the tested compound. Normalized data from each oocyte were fitted separately to the Hill equation (Prism 4.03; GraphPad, San Diego, CA, USA). EC_{50} or IC_{50} values obtained this way were averaged, and the S.E.M. was determined. Statistical significance was calculated as described above.

Determination of calcium permeabilities in *Xenopus laevis* oocytes

Calcium permeabilities were determined for GluR1(Q)flip in the presence and absence of TARPs. Serving as a standard (Soto et al., 1996), the calcium permeability of the KA receptor subunit GluR6(Q) was determined in parallel in the same preparation of oocytes. To avoid activation of endogenous Ca^{2+}-dependent Cl^- channels, oocytes in all experiments were injected with 50 nl of 200 mM EGTA, adjusted to pH 8.0 with NaOH, 20–30 min prior to