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Glycine release is regulated by metabotropic glutamate receptors sensitive to mGluR2/3 ligands and activated by *N*-acetylaspartylglutamate (NAAG)

Cristina Romei [a], Maurizio Raiteri [a,b,c], Luca Raiteri [a,b,*]

[a] Department of Experimental Medicine, Pharmacology and Toxicology Unit, University of Genoa, Genoa, Italy
[b] Center of Excellence for Biomedical Research, University of Genoa, Genoa, Italy
[c] National Institute of Neuroscience, Genoa, Italy

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ABSTRACT

The presence of metabotropic glutamate receptors (mGluRs) of group II modulating glycine exocytosis from glycinergic nerve endings of mouse spinal cord was investigated. Purified synaptosomes were selectively prelabeled with [^{3}H]glycine through the neuronal transporter GlyT2 and subsequently depolarized by superfusion with 12 mM KCl. The selective mGluR2/3 agonist LY379268 inhibited the K^+-evoked overflow of [^{3}H]glycine in a concentration-dependent manner (EC_{50} about 0.2 nM). The effect of LY379268 was prevented by the selective mGluR2/3 antagonist LY341495 (IC_{50} about 1 nM). *N*-acetylaspartylglutamate (NAAG) inhibited [^{3}H]glycine overflow with extraordinary potency (EC_{50} about 50 fmol). In contrast, glutamate was ineffective up to 0.1 nM, excluding that glutamate contamination of commercial NAAG samples is responsible for the reported activity of NAAG at mGluR3. LY341495 antagonized the NAAG inhibition of [^{3}H]glycine release. The effect of a combination of maximally effective concentrations of LY379268 and NAAG exhibited no additivity. The non-hydrolysable NAAG analogue *N*-acetylaspartyl-β-linked glutamate (β-NAAG) antagonized NAAG and LY379268. In conclusion, our results show that glycinergic nerve endings in spinal cord are endowed with group II mGluRs mediating inhibition of glycine exocytosis. NAAG can activate these presynaptic receptors with extremely high affinity and with characteristics compatible with the reported mGluR3 pharmacology.

This article is part of a Special Issue entitled 'Metabotropic Glutamate Receptors'.

1. Introduction

Glutamate is the agonist of two classes of receptors, ion channel-linked (ionotropic) receptors, which include NMDA, AMPA and kainate receptors, and metabotropic receptors (mGluRs) which couple via G-proteins to intracellular second messenger signalling pathways. The mGluRs are neuromodulatory receptors through which glutamate can regulate cell excitability and synaptic transmission. The widespread distribution of mGluRs in the CNS implies their participation in several functions and suggests that mGluRs may represent targets for therapeutic intervention in a variety of CNS pathologies (see, for recent reviews, Niswender and Conn, 2009; Nicoletti et al., 2011).

Genes encoding eight mGluR subtypes have been identified. Based on sequence homology, transduction mechanism and pharmacology, mGluRs are subdivided into three groups: group I includes mGluRs 1 and 5; group II includes mGluR2 and mGluR3 and group III includes mGluRs 4,6,7 and 8. Group II mGluRs are considered as potential targets for the treatment of acute and chronic neurodegenerative pathologies because agonists at these receptors have been shown to exhibit neuroprotective activity in models of excitotoxicity (see, for instance, Behrens et al., 1999; Bond et al., 1999; Battaglia et al., 2003; Corti et al., 2007 and references therein). Interestingly, experiments carried out with mGluR2 and mGluR3 knockout mice have shown that the neuroprotective activity towards NMDA of an agonist of group II receptors, LY379268, could be attributed to mGluR3 (Corti et al., 2007).

One major function of group II mGluRs is to negatively modulate neurotransmitter release from nerve endings on which mGluR2/3 are localized (reviewed in Niswender and Conn, 2009). Group II mGluRs exist on glutamatergic nerve terminals as presynaptic autoreceptors, activation of which brings about inhibition of glutamate exocytosis in different CNS regions including spinal cord

Abbreviations: β-NAAG, *N*-acetylaspartyl-β-linked glutamate; mGluRs, metabotropic glutamate receptors; NAAG, *N*-acetylaspartylglutamate.

* Corresponding author. Department of Experimental Medicine, Pharmacology and Toxicology Section, University of Genoa, Viale Cembrano 4, 16148 Genoa, Italy. Tel.: +39 010 3532659; fax: +39 010 3993360.
E-mail address: lraiter@pharmatox.unige.it (L. Raiteri).

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(Gerber et al., 2000) and various brain areas (Bushell et al., 1996; Conn and Pin, 1997; Raiteri, 2008). On the other hand, mGluR2/3 were shown to be also present as presynaptic heteroreceptors on GABAergic nerve terminals, their activation leading to a reduction in the release of GABA (Hayashi et al., 1993; Stefani et al., 1994; Petralia et al., 1996; Schaffhauser et al., 1998; Gerber et al., 2000).

If one assumes that inhibition of glutamate release onto NMDA receptors plays a major role in the neuroprotective activity of group II mGluR agonists, modulation of the release of glycine, the obligatory coagonist of glutamate at NMDA receptors, could be equally important. Yet, to the best of our knowledge, the existence on glycinergic terminals of mGluRs able to mediate inhibition of glycine exocytosis has only been suggested based on results from in vivo microdialysis in the periaqueductal grey (de Novellis et al., 2002) and from electrophysiology in spinal cord slices of nerve-injured animals (Zhou et al., 2011).

The present work was carried out with preparations of nerve endings purified from mouse spinal cord and prelabeled with [^{3}H]glycine. Release of [^{3}H]glycine was evoked by exposing synaptosomes during superfusion to a depolarizing concentration of KCl. Results obtained with selective group II mGluR ligands show that release-inhibiting receptors exist on spinal cord glycinergic nerve endings. Moreover, the sensitivity of these receptors to *N*-acetylaspartylglutamate (NAAG) seems consistent with the often reported (but recently challenged) ability of NAAG to activate receptors of the mGluR3 subtype.

2. Materials and methods

2.1. Animals

Adult male Swiss mice (weighing 20–25 g; Charles River, Calco, Italy) were used. Animals were housed at constant temperature (22 ± 1 °C) and relative humidity (50%) under a regular light/dark schedule (light 7.00 a.m to 7.00 p.m). Food and water were freely available. The experimental procedures were approved by the Ethical Committee of the Pharmacology and Toxicology Section, Department of Experimental Medicine, in accordance with the European legislation (European Communities Council Directive of 24 November 1986, 86/609/EEC). All efforts were made to minimize animal suffering and to use the minimum number of animals necessary to produce reliable results.

2.2. Preparation of synaptosomes

Animals were sacrificed and the spinal cord was quickly removed. The tissue was homogenized in 10 vol. of 0.32 M sucrose buffered at pH 7.4 with Tris–HCl, using a glass-teflon tissue grinder (clearance 0.25 mm, 24 up-down strokes in about 2 min). The homogenate was centrifuged (5 min, 1000 × *g* at 4 °C) to remove nuclei and debris and the supernatant was gently stratified on a discontinuous Percoll® gradient (2, 6, 10 and 20% v/v in Tris-buffered sucrose) and centrifuged at 33,500 × *g* for 5 min. The layer between 10% and 20% Percoll® (synaptosomal fraction) was collected, washed by centrifugation and resuspended in a physiological medium (standard medium) having the following composition (mM): NaCl, 140; KCl, 3; $MgSO_4$, 1.2; $CaCl_2$, 1.2; NaH_2PO_4, 1.2; $NaHCO_3$, 5; glucose, 10; HEPES, 10; pH adjusted to 7.4 with NaOH. All the above procedures were performed at 0–4 °C.

2.3. Experiments of release

Synaptosomes were incubated at 37 °C for 15 min with [^{3}H]glycine (0.05 μM) in the presence of the selective GLYT1 transporter blocker NFPS (0.3 μM). At the end of incubation, aliquots of the synaptosomal suspension (about 25 μg protein) were distributed on microporous filters placed at the bottom of a set of parallel superfusion chambers maintained at 37 °C (Superfusion System, Ugo Basile, Comerio, Varese, Italy) and superfused with standard medium, supplemented with 0.1% polypep®, at a rate of 0.5 ml/min (Raiteri and Raiteri, 2000; Popoli et al., 2012). After 36 min of superfusion with standard medium, to equilibrate the system, fractions were collected as follows: two 3-min samples (t = 36–39 and t = 45–48 min; basal release) before and after one 6-min sample (t = 39–45 min; evoked release). A 90-s period of depolarization was applied at t = 39 min. Depolarization of synaptosomes was performed with 12 mM KCl (substituting for an equimolar concentration of NaCl). LY379268, NAAG and glutamate were added concomitantly with KCl. LY341495 and β-NAAG were added 9 min before KCl. Fractions collected and superfused filters were counted for radioactivity by liquid scintillation counting.

2.4. Calculations

Neurotransmitter released in each fraction collected was expressed as a percentage of the radioactivity content of synaptosomes at the start of the respective collection period (fractional rate × 100). Depolarization-evoked neurotransmitter overflow was calculated by subtracting the transmitter content of the basal release from the transmitter content in the 6-min fraction collected during and after the depolarization pulse. Drug effects were evaluated by calculating the ratio of the depolarization-evoked neurotransmitter overflow in the presence of the drug versus that calculated under control conditions. Appropriate controls were always run in parallel.

2.5. Statistics

All data are given as means ± SEM. When appropriate, statistical comparison of data was performed by one-way ANOVA followed by *post-hoc* Dunnett's multiple comparison test. Differences were regarded as statistically significant for $p < 0.05$.

2.6. Materials

[^{3}H]Glycine (specific activity: 1.65 × 10^{15} Bq/mol) was purchased from Perkin Elmer (Boston, MA, USA). Percoll®, polypep®, NAAG, β-NAAG and glutamate were from Sigma Chemical Co. (St Louis, MO, USA). NFPS (also known as ALX 5407), LY379268 and LY341495 were purchased from Tocris Bioscience (Bristol, UK).

3. Results

Fig. 1 shows that the selective group II metabotropic glutamate receptor agonist LY379268 inhibited the [^{3}H]glycine overflow evoked by 12 mM KCl in a concentration-dependent manner (EC_{50} = 0.19 ± 0.02 nM; maximal inhibition ~50%, reached at 10 nM).

The effect of LY379268 was then tested in the presence of the selective group II metabotropic glutamate receptor antagonist LY341495. As shown in Fig. 2, LY341495 concentration-dependently blocked the inhibitory effect of LY379268 (1 nM), exhibiting IC_{50} about 1 nM. The effect of LY379268 was completely prevented when LY341495 was added at 30 nM. LY341495 did not significantly affect, on its own, the efflux of tritium (data not shown).

To investigate the involvement of receptors of the mGluR3 subtype, we tested the effect of NAAG on the K^+-evoked [^{3}H]glycine

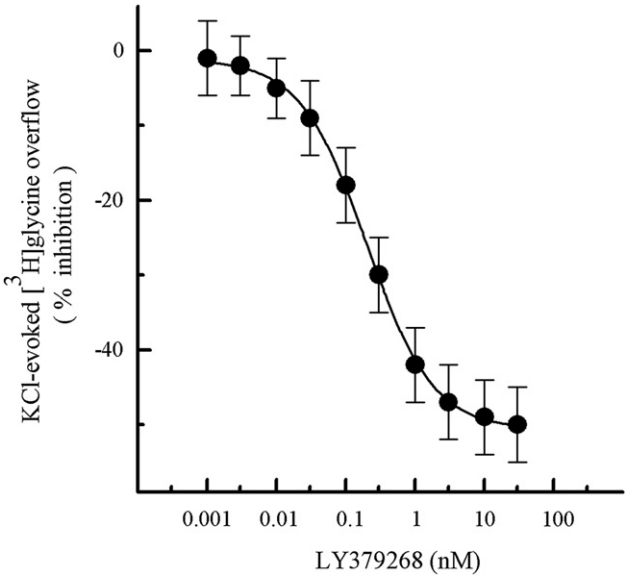

Fig. 1. Concentration-dependent inhibition of [^{3}H]glycine overflow from mouse spinal cord synaptosomes by LY379268. Synaptosomes were depolarized in superfusion with 12 mM KCl; LY379268 was added to the superfusion medium concomitantly with the depolarizing stimulus. Data are means ± SEM of 5 experiments in triplicate (three superfusion chambers for each experimental condition).