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2-Guanidine-4-methylquinazoline acts as a novel competitive antagonist of A type γ-aminobutyric acid receptors

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ABSTRACT

The pentameric A type γ-aminobutyric acid receptors ($GABA_A$Rs) are the major inhibitory neurotransmitter receptors in the nervous system and have long been considered as important pharmaceutical targets for the treatment of multiple neurological or psychological disorders. Here, we show that 2-guanidine-4-methylquinazoline (GMQ), a recently identified acid-sensing ion channel (ASIC) modulator, strongly and preferentially inhibits $GABA_A$R among the major neurotransmitter-gated ion channels in cultured rat hippocampal neurons. GMQ inhibited GABA (1 μM)-induced currents in a competitive manner, with an IC_{50} (0.39 ± 0.05 μM) comparable to that of bicuculline. Schild analysis revealed a slope of 1.04 ± 0.06 for GMQ on $\alpha_1\beta_2$ $GABA_A$Rs expressed in HEK293T cells. Single-channel analysis showed that GMQ decreased open probability of $GABA_A$Rs without affecting conductance. Moreover, GMQ inhibited GABAergic neurotransmission in hippocampal neurons, while having no significant effect on the basal field excitatory postsynaptic potentials (fEPSPs) and the intrinsic excitability of neurons. Using site-directed mutagenesis, we further demonstrated that mutations at Glu155 of β_2 subunit and Phe64 of α_1 subunit, both located inside the GABA binding pocket, profoundly decreased the sensitivity of the receptor to both GABA and GMQ. Interestingly, these mutations did not significantly affect the inhibition by amiloride, a diuretic structurally similar to GMQ and a known $GABA_A$R inhibitor. We conclude that GMQ represents a novel chemical structure that acts, possibly, by competing with GABA binding to $GABA_A$Rs. It is anticipated that GMQ and its analogs will facilitate the development of new chemical probes for $GABA_A$Rs.

1. Introduction

A type γ-aminobutyric acid receptors ($GABA_A$Rs) are pentameric chloride channels widely distributed in the central nervous system, mediating the primary inhibitory synaptic transmission and tonic inhibition (Macdonald and Olsen, 1994; Pirker et al., 2000). To date, 19 $GABA_A$R subunits have been identified: α (1–6), β (1–3), γ (1–3), δ (1), ε (1), π (1), θ (1), ρ (1–3). In addition, many of these subunits have splice variants, giving rise to a large repertoire of combinations, with rich complexity and variety in their physiological and

Abbreviations: GMQ, 2-guanidine-4-methylquinazoline; GABA, γ-aminobutyric acid; $GABA_A$R, A type γ-aminobutyric acid receptor; ASIC, acid-sensing ion channel; HBSS, Hank's Buffered Salt Solution; DMEM, Dulbecco's Modified Eagle's Medium; ACSF, artificial cerebrospinal fluid; Bic, bicuculline-methobromide; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; D-AP5, D(−)-2-amino-5-phosphonopentanoic acid; TTX, tetrodotoxin; HEPES, N-hydroxyethylpiperazine-N-2-ethanesulphonic acid; I-V, current-voltage; mIPSC, miniature inhibitory postsynaptic current; fEPSPs, field excitatory postsynaptic potentials; PS, population spike; DR, dose ratio; GLIC, Gloeobacter ligand-gated ion channel; AMPAR, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; NMDAR, N-methyl-D-aspartic acid receptor; ACh, acetylcholine; nAChR, nicotinic acetylcholine receptor.

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pharmacological properties (Enna and Mohler, 2007; Olsen and Sieghart, 2008). Despite these varieties, the main subunit combination is $\alpha_1\beta_2\gamma_2$, at a ratio of 2:2:1, which makes up 43% of the $GABA_A$Rs in the adult brain (McKernan and Whiting, 1996).

Being responsible for inhibitory tone in the central nervous system and thus controlling the balance between excitation and inhibition, $GABA_A$Rs are involved in almost every aspect of the normal brain function. Either excessive or insufficient $GABA_A$R activity may lead to a variety of diseases and disorders including insomnia, epilepsy, anxiety, depression or even death. As a result, $GABA_A$Rs have long been considered as important pharmaceutical targets. Although most of the $GABA_A$R-targeted drugs in clinical use, such as benzodiazepines, barbiturates, steroids and anesthetics, are positive modulators of $GABA_A$Rs, there is growing evidence that low doses of $GABA_A$R antagonists or negative modulators have therapeutic potentials in diseases where excessive inhibitory tone exists, such as in Down syndrome (Fernandez et al., 2007; Rueda et al., 2008). Therefore, continuing efforts are made to develop or discover novel $GABA_A$R negative modulators.

Here, we report 2-guanidine-4-methylquinazoline (GMQ) as a novel inhibitor of $GABA_A$Rs. GMQ is a small molecule with a hydrophilic guanidine group directly attached to a hydrophobic quinazoline structure. It was first reported to have anti-secretory effects in the gastric system and anti-histamine effects in isolated guinea pig auricle and rat uterus (Pinelli et al., 1996). In our previous work, we found that GMQ directly activated acid-sensing ion channel type 3 (ASIC3) at millimolar concentrations through the nonproton sensor domain and elicited pain-related behaviors in mice (Yu et al., 2010). A more recent work by another group further characterized its effect on other ASIC subtypes (Alijevic and Kellenberger, 2012). However, the concentrations of GMQ used in these studies were very high and as such it might affect other ion channels or receptors. In this work, we tested the effects of GMQ on several ligand-gated ion channels and found that GMQ strongly and preferentially inhibited $GABA_A$Rs at micromolar concentrations in cultured hippocampal neurons. We then further characterized the pharmacological properties and molecular basis of GMQ inhibition on $GABA_A$Rs.

2. Materials and methods

2.1. Animals

Animals used in this study were handled and housed in accordance with the guidelines and protocols approved by the Animals Care and Use Committee of the Institute of Neuroscience, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. All efforts were made to minimize animal suffering and to reduce the number of animals used.

2.2. Expression vectors and site-directed mutagenesis

cDNA constructs encoding the rat $GABA_A$R α_4, α_5, β_2 and γ_2L subunits were cloned into pcDNA3.1 vector and α_1 in pCDM8 vector. Point mutations were generated using the QuickChange Mutagenesis kit (Stratagene) with purified oligonucleotide primers. The coding regions of all mutant constructs were verified by DNA sequencing (Shanghai Sunny Biotechnology Co, Ltd, Shanghai).

2.3. Cell culture and transfection

Hippocampal neurons from embryonic day 18 (E18) of Sprague–Dawley (SD) rats were isolated as previously described (Gao et al., 2005). Briefly, rat hippocampi were dissected in Hank's Buffered Salt Solution (HBSS) and digested with 0.25% trypsin at 37 °C for 10 min. The dissociated neurons were then plated onto poly-D-lysine coated glass coverslips in Dulbecco's Modified Eagle's Medium (DMEM) plus 10% F12 medium and 10% fetal bovine serum. A half of the culture medium was replaced by Neurobasal Medium supplemented with 1% glutamine and 2% B27 the next day and every 3–4 days thereafter. At three days after plating, cultures were treated with 5-fluoro-5′-deoxyuridine (20 μg/ml, Sigma, St. Louis, MO) to block the excessive proliferation of non-neuronal cells. The neurons were maintained at 95% air and 5% CO_2 humidified atmosphere and used at 10–16 days after plating.

Human embryonic kidney (HEK) 293T cells were maintained in DMEM supplemented with 10% fetal bovine serum and passaged every 3–4 days. For transient transfection, cells were plated onto 35-mm dishes and transfected the next day using the HilyMax transfection reagent (Dojindo Molecular Technologies, Inc. Kumamoto, Japan). Plasmids for the rat $GABA_A$R α_1 and β_2 subunits or mutants were added at 1:1 ratio of 2 μg each plus 1 μg pEGFP-C3 plasmid for visualization of the transfected cells by green fluorescence. For the $\alpha_1\beta_2\gamma_2$ recombinant receptor, the plasmid ratio was 1:1:6. Functional expression of the $\alpha_1\beta_2\gamma_2$ receptor was confirmed using Zn^{2+} because of its relative insensitivity to zinc blockade (Draguhn et al., 1990; Gingrich and Burkat, 1998; Smart et al., 1991). A block of less than 15% of the current by 1–2 μM Zn^{2+} indicates channel composition of mainly $\alpha_1\beta_2\gamma_2$. After 16–24 h, the transfected cells were trypsinized and plated onto glass coverslips for further experiments.

2.4. Brain slice preparation

Transverse brain slices encompassing hippocampal regions (400 μm thick) were prepared from 14 to 21-day-old male SD rats as described previously (Gong et al., 2008). After decapitation, the brain was quickly removed and placed into oxygenated ice-cold artificial cerebrospinal fluid (ACSF) bubbled with a mixture of 95% O_2 plus 5% CO_2. The ACSF contained (in mM): 119 NaCl, 2.5 KCl, 2.5 $CaCl_2$, 1 NaH_2PO_4, 1.3 $MgSO_4$, 26.2 $NaHCO_3$ and 10 D-glucose, saturated with 95% O_2/5% CO_2 (pH 7.4), and was used throughout the experiment. Slices were cut using a Leica VT1000S vibratome (Leica instruments Ltd., Wetzlar, Germany). After incubation in continuously oxygenated ACSF at 28 °C for 1–2 h, the slice was transferred to the recording chamber mounted on an upright microscope (BX51WI, Olympus Corporation, Tokyo, Japan) and was gently held between two nylon nets and continuously perfused with oxygenated ACSF at room temperature (23–25 °C). The flow rate of the perfusion during recording was 2–2.5 ml/min. Recordings were performed on CA1 pyramidal neurons or stratum radiatum at room temperature.

2.5. Electrophysiological recordings

All electrophysiological recordings were performed using the Axopatch amplifier, either 200B (for brain slices and single-channel recordings) or 700A (for cultured cells) (Axon Instruments, Foster City, CA). Data were acquired and analyzed by the Digidata 1320A interface and pClamp9.0 software. Currents were low-pass filtered at 2 kHz and sampled at 10 kHz. The pipettes were pulled from 1.5 mm borosilicate glass capillary on a vertical two-stage puller (PP-830, Narishige Co., Ltd., Tokyo, Japan). For extracellular field recordings, a bipolar platinum–iridium stimulating electrode was placed at the Shaffer collateral axons and a stimulus (0.1 ms) was given every 30 s. Recording pipettes were filled with ACSF (2.5–4 MΩ) and placed in the stratum radiatum and CA1 cell body layer to record field excitatory postsynaptic potential (fEPSP) and population spike (PS), respectively. Stimulation intensity was adjusted to generate 30–40% of the maximal response. PS amplitude was calculated as the difference between the negative peak and the estimated baseline value at the same time point based on linear interpolation of the two flanking positive peaks (Gong et al., 2008).

Whole-cell recordings were performed in CA1 pyramidal neurons for action potentials and evoked inhibitory postsynaptic currents (eIPSCs). The intracellular solution for recording action potentials contained (in mM): 140 K gluconate, 5 NaCl, 2 MgATP, 0.2 EGTA, and 10 HEPES. The pH was adjusted to 7.2 with KOH and the osmolarity was ~290 mOsm. To eliminate the influence of synaptic transmission on action potentials, 10 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), 40 μM D-AP5 and 20 μM bicuculline-methobromide (Bic) were added to the ACSF for at least 5 min before application of GMQ. In current recording mode, action potentials were elicited by applying 500 ms current (ranging from −100 pA to +450 pA at 50-pA steps and 5-s intervals). For voltage clamp recordings of IPSCs, the membrane potential was held at −70 mV throughout the experiment and the intracellular solution was the same as above, except that K gluconate was replaced with equivalent CsCl and 2.5 mM QX-314 was added intracellularly to block action potentials. To record evoked IPSCs, a bipolar platinum–iridium stimulating electrode was placed at the Shaffer collateral axons 300–400 μm away from the recording pipette. Drugs were bath-applied and their effects were measured at least 5 min after drug application or washout.

For electrophysiological recordings in cultured neurons and HEK293T cells, the standard extracellular solution contained (in mM): 150 NaCl, 5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 HEPES, 10 D-glucose, pH adjusted to 7.4 with Tris base. The osmolarity was adjusted to 320–330 mOsm. The intracellular solution contained (in mM): 120 CsCl, 30 NaCl, 1 $MgCl_2$, 0.5 $CaCl_2$, 5 EGTA, 2 MgATP, and 10 HEPES, pH adjusted to 7.2. To record acetylcholine (ACh)-evoked currents, NaCl was replaced by 20 mM phosphocreatine disodium (Alkondon et al., 1994). Pipette resistance was 3–4 MΩ for whole-cell recordings and 6–10 MΩ for single-channel recordings. Drugs were applied by gravity through a "Y-tube", placed about 700 μm away from the cell being recorded. This drug application system allows rapid and complete exchange of extracellular solution within 20 ms (Murase et al., 1990). In addition, fresh external solution driven by gravity was perfused through the culture dish at 2 ml/min to washout the residual drug. When miniature IPSCs (mIPSCs) were recorded in cultured hippocampal neurons, 0.3 μM tetrodotoxin (TTX), 10 μM CNQX and 40 μM D-AP5 were added to the bath. All whole-cell recordings were performed at least 5 min after break-in and the stability was monitored by Clampex's Membrane Test. The access resistance measured was <10 MΩ for HEK293T cells and <25 MΩ for