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Cyclothiazide selectively inhibits mGluR1 receptors interacting with a common allosteric site for non-competitive antagonists

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Abstract

Metabotropic glutamate receptors mGluR1 and mGluR5 stimulate phospholipase C, leading to an increased inositol trisphosphate level and to Ca^{2+} release from intracellular stores. Cyclothiazide (CTZ), known as a blocker of AMPA receptor desensitization, produced a non-competitive inhibition of $[Ca^{2+}]_i$ increases induced by mGluR agonists in HEK 293 cells transfected with rat mGluR1a but had no effect on the $[Ca^{2+}]_i$ signals in cells expressing rat mGluR5a. In cells expressing mGluR1, CTZ also inhibited phosphoinositide hydrolysis, as well as cAMP accumulation and arachidonic acid release induced by mGluR1 agonists, indicating a direct inhibition of the receptor and not of a particular signal transduction system. However, CTZ failed to antagonize cAMP inhibition stimulated by rat mGluR2, -3, -4, -6, -7 and -8 receptors confirming its selectivity for mGluR1. The use of chimeric receptors with substituted N-terminal domains showed that CTZ did not interact with the N-terminal mGluR1a domain. Instead, mutation analysis revealed that CTZ interacts with the Thr-815 and Ala-818 residues, located at the 7th transmembrane domain, similarly as the mGluR1-selective antagonist CPCCOEt. In primary cultures of cerebellar granule neurons, expressing native metabotropic and ionotropic glutamate receptors, the final outcome of CTZ effects depended on its combined ability to potentiate AMPA receptors and inhibit mGluR1 receptors.

Keywords: Metabotropic glutamate receptors; Cyclothiazide; Non-competitive antagonist; Cerebellar granule cells

1. Introduction

Metabotropic glutamate receptors (mGluRs) belong to the large family of G-protein-coupled receptors. Eight mGluRs subtypes and multiple splice variants have been identified and classified into three groups on the basis of sequence similarities, second-messenger coupling and pharmacological properties. Group I receptors (mGluR1 and -5) are coupled to the stimulation of phospholipase C (PLC), which results in the hydrolysis of membrane phosphoinositides (PI) followed by increased Ca^{2+} release from intracellular stores. In addition, when expressed in several cellular systems, mGluR1a can also enhance the formation of cAMP and the release of arachidonic acid (Aramori and Nakanishi, 1992). Group II (mGluR2 and -3) and III (mGluR4, -6, -7 and -8) receptors are negatively coupled to adenylyl cyclase and decrease cAMP accumulation in heterologous expression systems (Conn and Pin, 1997; De Blasi et al., 2001). All mGluRs share structural similarities, which include a large N-terminal extracellular domain, seven transmembrane (TM) spanning regions, and a variable-length C-terminal domain (Jingami et al., 2003; Bhave et al., 2003).

Group I mGluR antagonists include competitive antagonists, which bind to the N-terminal receptor domain and non-competitive antagonists interacting within the TMVII domain (Gasparini et al., 2002). The first reported selective non-competitive mGluR1 antagonist 7-hydroxyiminocyclopropan[*b*]chromen-1a-carboxylic acid ethyl ester (CPCCOEt), was shown to interact with the Thr-815 and Ala-818 residues, located at the extracellular surface of TMVII (Litschig et al., 1999). Another potent selective non-competitive mGluR1 antagonist (3aS,6aS)-6a-naphtalen-2-ylmethyl-5-methyliden-hexahydro-cyclopental [*c*]furan-1-on (BAY36-7620) was also reported to interact with

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the TM region of the receptor (Carroll et al., 2001). Recently a selective non-competitive mGluR1 antagonist 1-ethyl-2-methyl-6-oxo-4-(1,2,4,5-tetrahydro-benzo[*d*]azepin-3-yl)-1,6-dihydro-pyrimidine-5-carbonitrile (EM-TBPC) was shown to bind within the TM domains, however, residues Val-757, Trp-798, Phe-801, Tyr-805 and Thr-815 are critical determinants of the EM-TBPC binding pocket of mGluR1 (Malherbe et al., 2003). Another study showed that the binding of a new potent non-competitive mGluR1 receptor-selective antagonist [^{3}H]1-(3,4-dihydro-2*H*-pyrano[2,3-*b*]quinolin-7-yl)-2-phenyl-1-ethanone (R214127) was completely blocked by 2-quinoxaline-carboxamide-*N*-adamantan-1-yl (NPS 2390), BAY 36-7620 and CPCCOEt, but was not displaced by competitive mGluR1 ligands such as glutamate and quisqualate, suggesting that R214127, NPS 2390, BAY 36-7620, and CPCCOEt bind to the same site or to mutually exclusive sites (Lavreysen et al., 2003). A recently discovered selective non-competitive mGluR1 antagonist 6-amino-*N*-cyclohexyl-*N*,3-dimethylthiazolo[3,2-*a*]benzimidazole-2-carboxamide (YM-298198) has also been shown to bind to the CPCCOEt allosteric binding site (Kohara et al., 2005). In addition, a new class of non-competitive mGluR1 antagonists, 2,4-dicarboxy-pyrroles were shown to interact within the TMVII domain of the receptor (Micheli et al., 2003). In contrast, the mGluR5 selective antagonist 2-methyl-6-(phenylethynyl)pyridine (MPEP) binds to different non-conserved residues, Pro-655 and Ser-658 in TMIII and Ala-810 in TMVII.

Our findings indicate that, in addition to the non-competitive antagonists mentioned above, mGluR1 can be inhibited by cyclothiazide (CTZ). CTZ is known to enhance the activity of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors by suppressing their desensitization (Partin et al., 1993, 1995). Moreover, CTZ inhibits γ-aminobutyric acid type A receptors ($GABA_A$) having no effect on GABA binding (Deng and Chen, 2003). In addition, in *Xenopus* oocytes injected with mRNA for group I mGluRs, CTZ reversibly blocked mGluR1a-mediated, Ca^{2+}-dependent chloride currents but slightly potentiated mGluR5a-mediated currents (Sharp et al., 1994). The inhibition of mGluR1a receptors expressed in oocytes was non-competitive; however the site of CTZ action has not been identified.

The aim of this study was to characterize the ability of CTZ to modulate agonist-induced intracellular $[Ca^{2+}]_i$ signals and PI hydrolysis in cells expressing rat group I mGluRs (mGluR1a and mGluR5a). Moreover, we have determined that CTZ binds to the same binding site as the selective non-competitive mGluR1 antagonist CPCCOEt.

2. Materials and methods

2.1. Materials

Fura-2/AM and Pluronic F-127 were obtained from Molecular Probes, Inc. (Eugene, OR) and ionomycin from Calbiochem (San Diego, CA). Neurobasal culture media, B27 supplement and fetal bovine serum for neuronal cultures were purchased from Gibco-BRL (Gaithersburg, MD). All restriction enzymes were from New England Biolabs (Beverly, MD). EMEM, DMEM and fetal bovine serum for transfected cell cultures were purchased from Biofluids (Rockville, MD). L-Quisqualic acid, (1*S*,3*R*)-1-aminocyclopentane-1,3-dicarboxylic acid (ACPD), CTZ, CPCCOEt, (2*S*,1′*S*,2′*S*)-2-(carboxycyclopropyl)glycine (L-CCG-I), (±)-2-amino-4-phosphonobutyric acid (AP-4), 1-(4′-aminophenyl)-3,5-dihydro-7,8-dimethoxy-4*H*-2,3-benzodiazepin-4-one (CFM2), 2,3-dihydro-6-nitro-7-sulfamoyl-benzo[*f*]quinoxaline (NBQX) and (*RS*)-4-carboxy-3-hydroxyphenylglycine (4C3HPG) were obtained from Tocris Cookson, (Ellisville, MO). All other chemicals were from Sigma (St. Louis, MO). Ionomycin, CTZ, and CPCCOEt were dissolved in 100% DMSO and appropriate amounts of solvent were added to all controls.

2.2. Preparation of transfection vectors

Rat mGluR cDNAs were cloned into the mammalian expression vector pcDNA3.1 (Invitrogen, Carlsbad, CA) under control of the CMV IE promoter/enhancer. This vector contains neomycin resistance gene allowing stably transfected cells to be selected using G-418 (Mediatech, Herndon, VA). Chimeric molecules containing the extracellular domains of either mGluR2 or mGluR3 and the transmembrane domains and C-terminus of mGluR1a were prepared using the PCR-based overlap extension method (Horton et al., 1993). The primers, which shared complementary sequence on the strands to be joined were designed as follows: 5′-GTA CAT CCG CTG GGG TGA TAT AGA ATC TAT CAT AGC C-3′ (primer A, mGluR2-mGlur1a), 5′-GGC TAT GAT AGA TTC TAT ATC ACC CCA GCG GAT GTA C-3′ (primer B, mGluR1a-mGluR2), and 5′-TTA CAT CAA ATG GGA AGA CAT AGA ATC TAT CAT AGC C-3′ (primer C, mGluR3-mGluR1a), and 5′-GGC TAT GAT AGA TTC TAT GTC TTC CCA TTT GAT GTA A-3′ (primer D, mGluR1a-mGluR3). To obtain the 5′-end of the chimeric molecule, mGluR2 cDNA was PCR-amplified with T7-primer and primer B, while for the 3′-end, SP6-primer and primer A were used. To obtain the 5′-end and 3′-end of mGluR3/mGluR1a chimeric molecules, the primer pairs T7 and primer D, and SP6 and primer C were used, respectively. The reaction mixture contained 2 mM $MgCl_2$, 1 μM primers, 200 μM dNTPs, 5 units of *Pfu* DNA polymerase (Stratagene, La Jolla, CA) and 100 ng of DNA-template in a volume of 100 μl. After denaturation at 94 °C for 1 min 30 cycles were performed: denaturation at 94 °C for 45 s, annealing at 50 °C for 2 min, extension at 72 °C for 10 min. The PCR-products were purified on LMP-agarose and amplified using PCR primers: T7 and SP6. Amplified products were digested with *Hind*III and *Not*I restriction enzymes, purified on LMP-agarose, and cloned into the pcDNA3.1 expression vector.

2.3. Cell cultures

Human embryonic kidney (HEK 293) cells were transiently transfected using Effectene transfection reagent (Qiagen, Valencia, CA). Cells were grown in DMEM supplemented with 10% fetal bovine serum and 2 mM glutamine in CO_2 (6%) incubator. Cells were used for intracellular Ca^{2+} measurements 24–72 h after transfection. Chinese hamster ovary (CHO-K1) or baby human kidney (BHK) cells were used for stable expression of mGluRs. Individual cell lines were isolated and cultured in DMEM supplemented with 10% fetal bovine serum, 1% of L-proline, 2 mM glutamine and 0.8 mg/ml G-418 in CO_2 (6%) incubator. Primary cultures were prepared as described previously for rat cerebellar granule cells (Wroblewski et al., 1985) and rat cortical astrocytes (Pshenichkin and Wise, 1995). Neuronal cultures were maintained in neurobasal medium supplemented with B27 and 2 mM glutamine, 100 μg/ml gentamicin, and either 5 or 25 mM KCl. To prevent the growth of non-neuronal cells cytosine arabinoside (10 μM) was added next day after plating. Astrocytes were cultured in EMEM medium supplemented with 10% fetal bovine serum.

2.4. Site-directed mutagenesis

Introduction of point mutations in mGluR1a cDNA was made using the QuikChange Site-Directed Mutagenesis Kit (Stratagene). Briefly, 20 ng of plasmid containing mGluR1a cDNA were mixed with 125 ng of two mutagenic primers, dNTPs (50 μM) and 2.5 units of *Pfu* DNA polymerase in a final volume of 50 μl. Mutagenic primers (forward primer: 5′-CTA CAA GAT CAT