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Native metabotropic glutamate receptor 4 depresses synaptic transmission through an unusual $G\alpha_q$ transduction pathway

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ABSTRACT

In cerebellar cortex, $mGlu_4$ receptors located on parallel fibers play an essential role in normal motor function, but the molecular mechanisms involved are not yet completely understood. Using a strategy combining biochemical and electrophysiological approaches in the rodent cerebellum, we demonstrate that presynaptic $mGlu_4$ receptors control synaptic transmission through an atypical activation of $G\alpha_q$ proteins. First, the $G\alpha_q$ subunit, PLC and PKC signaling proteins present in cerebellar extracts are retained on affinity chromatography columns grafted with different sequences of the cytoplasmic domain of $mGlu_4$ receptor. The i2 loop and the C terminal domain were used as baits, two domains that are known to play a pivotal role in coupling selectivity and efficacy. Second, *in situ* proximity ligation assays show that native $mGlu_4$ receptors and $G\alpha_q$ subunits are in close physical proximity in cerebellar cortical slices. Finally, electrophysiological experiments demonstrate that the molecular mechanisms underlying $mGlu_4$ receptor-mediated inhibition of transmitter release at cerebellar Parallel Fiber (PF) – Molecular Layer Interneuron (MLI) synapses involves the $G\alpha_q$-PLC signaling pathway.

Taken together, our results provide compelling evidence that, in the rodent cerebellar cortex, $mGlu_4$ receptors act by coupling to the $G\alpha_q$ protein and PLC effector system to reduce glutamate synaptic transmission.

1. Introduction

Metabotropic glutamate receptors (mGlu receptors) are G-protein coupled receptors that modulate fast excitatory and inhibitory transmission throughout the central nervous system. mGlu receptors form a 8 member family that is divided into three groups (I, II and III) based on receptor agonist affinity, sequence homology and second messenger signaling pathway selectivity (Pin and Duvoisin, 1995; Conn and Pin, 1997). For the most part, mGlu receptor responses involve the activation of G-proteins. Studies carried out essentially in heterologous expression systems have shown that Group I mGlu receptors are positively coupled to $G\alpha_q/G\alpha_{11}$, whereas all other receptor subtypes are coupled to $G\alpha_{i/o}$ (Nicoletti et al., 2011). However, mGlu receptors can also act independently of G-proteins through diverse intracellular interactions (Gerber et al., 2007). Although mGlu receptor studies have provided better understanding of their mechanisms of action, for certain receptor subtypes, the intracellular signaling pathways downstream to native mGlu receptor activation remain to be characterized.

Subtypes 4, 7 and 8 of Group III mGlu receptors are of particular interest since they are predominantly located on presynaptic terminals where they inhibit glutamate (autoreceptors) and/or GABA

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(heteroreceptors) synaptic transmission (Ferraguti and Shigemoto, 2006; Mercier and Lodge, 2014). This inhibition is mostly attributed to their regulation of presynaptic ion channels: inhibition of voltage-gated Ca^{2+} channels (VGCC) and activation of various K^+ channels (Anwyl, 1999). In addition, group III mGlu receptor activation can directly affect exocytosis (Chavis et al., 1998). Through their conventional coupling to $G\alpha_{i/o}$, these receptors have been shown to inhibit adenylyl cyclase (AC), and thus cAMP formation in heterologous expression systems (Nicoletti et al., 2011). However a few studies with native receptors show non-conventional signaling pathways downstream of the G protein. Indeed, Lavialle-Defaix et al. (2006) have shown that in the cockroach central nervous system, group III mGlu receptors couple with $G\alpha_s$ proteins, leading to AC activation and increases in cAMP levels. In cerebellar granule cell cultures, native group III mGlu receptors are functionally coupled to both mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3 kinase (PI3K) pathways (Iacovelli et al., 2002).

$mGlu_4$ receptors are expressed presynaptically on parallel fibers (PF). $mGlu_4$ receptor knock-out mice display altered short-term synaptic plasticity and poor performance in rotating rod motor learning. This suggests that the cerebellar function of $mGlu_4$ receptor supports learning of complex motor tasks through the control of synaptic efficacy. Indeed, our group, along with others, has demonstrated that $mGlu_4$ receptor activation decreases glutamate release at least in part by inhibiting VGCCs, leading to a reduction in evoked calcium influx (Daniel and Crepel, 2001; Lorez et al., 2003; Abitbol et al., 2008). This inhibition requires an intracellular signaling pathway that involves both Phospholipase C (PLC) and Protein Kinase C (PKC) at Parallel Fiber – Purkinje Cell (PF-PC) synapses (Abitbol et al., 2012).

Building on these results, we asked whether this pathway is restricted to the PF-PC synapse or is more widely involved in the $mGlu_4$ receptor depressant effect. Thus, in the present study we undertook a strategy that combines biochemistry, cell biology and electrophysiology methods. We performed affinity chromatography experiments using several cytoplasmic domains of $mGlu_4$ receptor, such as the i_2 loop and its C terminal domain, respectively known to play a pivotal role in the selectivity and efficacy of G-protein coupling (Gomeza et al., 1996; Havlickova et al., 2003). Through mass spectrometry analysis and/or western blots, we show that these intracellular domains can interact with a large number of proteins issued from whole cerebellar extracts, such as $G\alpha_q$, PLC and PKC. Using an *in situ* Proximity Ligation Assay, we also demonstrate that native $mGlu_4$ receptor and $G\alpha_q$ are in close proximity in the cerebellar cortex. Finally, our electrophysiological experiments show that $mGlu_4$ receptor reduces glutamatergic transmission at PF–molecular layer interneuron (MLI) synapses, through a mechanism that also employs the protein $G\alpha_q$ - PLC pathway.

2. Materials &methods

2.1. Animals

Animal care and euthanasia procedures were in accordance with European legislation. Male Sprague Dawley rats and C57BL/6 wild-type mice came from Janvier Laboratories (Le Genest-St-Isle, France). Male mutant mice lacking the $mGlu_4$ receptor (C57BL/6 background) were purchased from Jackson Laboratories (Bar Harbor, ME), with Charles River Laboratories (Saint Germain sur l'Arbresle, France) as the international import and distribution agent.

2.2. Material

The following antibodies were used in western blotting and Proximity Ligation Assay (PLA) experiments: $mGlu_4$ receptor (ab51-3100, rabbit polyclonal, 5 mg/mL, Invitrogen, Carlsbad, NM, USA), anti-PLC beta-1 (SC-205, Santa Cruz, France), anti-PLC beta-4 (SC-404, Santa Cruz), anti-PKC gamma (SC-211, Santa Cruz), anti GNAQ/GNA11 (ab79337, goat polyclonal, 5 mg/mL, Abcam), anti-adenylyl cyclase 1 (C12031, Assay bioTech), anti-PKA reg (H90, sc-28893, Santa Cruz), anti PKA cat (C20, sc-903, Santa Cruz), and anti-MAP kinases ERK1+ERK2 (13-6200, Invitrogen), anti-$G_{i/o}$ protein (goat polyclonal, 1/50, Santa Cruz), anti-G_s protein (mouse monoclonal, 1/50, Santa Cruz). Triton X-100 was purchased from Sigma-Aldrich (St Louis, MO, USA), the antiprotease cocktail from Roche (Basel, Switzerland) and modified trypsin from Promega (Madison, WI, USA).

L-AP4 (L-(+)-2-amino-4-phosphonobutyric acid), U73122 (1-[6-[[(17β)-3-Methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1H-pyrrole-2,5-dione), M-SOP ((RS)-α-methylserine-O-phosphate), PHCCC (*N*-phenyl-7-(hydroxylimino)cyclopropa[*b*]-chromen-1a-carboxamide), TTX (tetrodotoxin) and D-AP5 ((D)-(-)-2-amino-5-phosphonopentanoic acid) were purchased from Tocris (Illkirch, France). BAPTA-AM 1,2-Bis(2-aminophenoxy)ethane-N,N,N′,N'-tetraacetic acid tetrakis(acetoxymethyl ester) was purchased from Abcam (France). UBO-QIC was purchased from the institute of Pharmaceutical Biology (University of Bonn, Germany). U7343 (1-[6-[[(17β)-3-Methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1H-pyrrole-2,5-dione) and bicuculline methiodide were purchased from Sigma. All drug stocks were prepared in distilled water, except for Fluo4FF-AM (Molecular Probes), PHCCC, U73122 and U73343, which were prepared in dimethylsulfoxide (DMSO, final concentration ≤ 0,1%). Drug stocks were kept at −20 °C until use.

2.3. Protein extraction

30-37 day-old male Sprague-Dawley rats were used. Animals were stunned and then decapitated. The cerebellum was homogenized using a potter in a buffer solution containing (in mM) NaCl, 50; HEPES-NaOH pH 8.0, 25; EDTA, 0.5; and 0.25 mg/mL AEBSF, 0.01 mg/mL E-64, 0.05 mg/mL Antipaïn, 1 mM Na_3VO_4 and an anti-phosphatase cocktail (Sigma-Aldrich) with a ratio of 100 μL/10 mg of tissue. The homogenate was centrifuged at 14000 × g for 20 min at 4 °C. The supernatant was centrifuged at 100000 × g for 1 h at 4 °C and the small resulting pellet was discarded, while the supernatant was recovered for the experiments (see below Soluble protein fraction (S)). The pellet from the 14000 × g centrifugation was solubilized in the above buffer supplemented with 1% Triton X-100 (v/v) and centrifuged again at 14000 × g for 20 min at 4 °C. The supernatant constitute is what we hereafter call the membrane cerebellar protein (M) fraction and contains a large part of the solubilized membrane proteins. Both extracts were used for affinity chromatography experiments.

2.4. Affinity chromatography with peptides corresponding to $mGlu_4$ receptor cytoplasmic parts

For affinity chromatography experiments, 5 peptides issued from the intracellular part of $mGlu_4$ receptor were synthesized (GeneCust, Luxembourg): the nearly full length C-terminal peptide named «C-ter» (59 out of 72 aa) (AKR KRSLKAVV-TAATMSNKFTQKGNFRPNGEAKSELCENLETPALATKQTYVTYTNHAI), three truncated cytoplasmic parts, named «C1» (AEQNVPKRKRSL-KAVVTAATMSN), «C2»(VVTAATMSNKFTQKGNFRPNGEAKSELCEN), «C3» (GEAKSELCENLETPALATKQTYVTYTNHAI), and the second intracellular loop «i_2 loop» (NRIYRIFEQGKRSVSAPRFISPASQ).