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C-terminal deletion of metabotropic glutamate receptor 1 selectively abolishes coupling to Gαq

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ABSTRACT

Recent studies indicate that the intracellular C-terminus of Group I metabotropic glutamate receptors ($mGlu_1$ and $mGlu_5$ receptor) is important in G protein coupling. To determine the necessity of the C-tail, a deletion mutant of $mGlu_1$ receptor was constructed, which included the first 840 amino acids of the rat $mGlu_{1a}$ receptor ($mGlu_1$-dCT). G protein coupling of the receptors was assessed by measuring glutamate mediated inhibition of native calcium currents when each receptor was expressed in isolated sympathetic neurons from the rat superior cervical ganglion. Wild type $mGlu_1$ receptor activates both the $G\alpha_{i/o}$ and $G\alpha_{q/11}$ protein families. Each pathway can be detected in superior cervical ganglion neurons as voltage dependent and voltage independent inhibition of the calcium currents, respectively. While wild type $mGlu_1$ receptor gave rise to a strong, mixed voltage dependent and independent calcium current inhibition, $mGlu_1$-dCT exhibited a weaker inhibition that was strongly voltage dependent, indicating activation of $G\alpha_{i/o}$ was predominant. Further, pertussis toxin treatment reduced the inhibition by wild type $mGlu_1$ receptor to a smaller, voltage independent inhibition as expected, but completely abolished signaling through $mGlu_1$-dCT. Finally, to test whether $mGlu_1$-dCT could produce any activation of $G\alpha_{q/11}$, inhibition of the native superior cervical ganglion M-type potassium currents was examined. M-channels, inhibited by PIP_2 depletion, were strongly inhibited by glutamate in cells expressing wild type $mGlu_1$ receptor, but no inhibition was detectable in neurons expressing $mGlu_1$-dCT. These data indicate that C-terminal deletion of $mGlu_1$ receptor selectively abolishes $G\alpha_{q/11}$ coupling.

1. Introduction

Metabotropic glutamate receptors (mGlu receptors) are members of the Class C of the G protein coupled receptor superfamily. Class C receptor shares the basic heptahelical topology of the more thoroughly studied, rhodopsin-like Class A receptors, but differ in sequence homology, their mechanism of activation, and in the regions responsible for G protein coupling. The group I mGlu receptors ($mGlu_1$ and $mGlu_5$) are unique among the mGlu receptor family in that they can couple to multiple G protein classes (Hay and Kunze, 1994; Kammermeier and Ikeda, 1999; McCool et al., 1998). In most cases, coupling to $G\alpha_{q/11}$ is observed and is coincident with activation of either $G\alpha_s$ or $G\alpha_{i/o}$ when the receptors function in heterologous expression systems or in neurons, respectively (Aramori and Nakanishi, 1992; Francesconi and Duvoisin, 2000; Hay and Kunze, 1994; Joly et al., 1995; Kammermeier and Ikeda, 1999).

Recent studies have implicated the C-tail of $mGlu_1$ receptor as a regulator of G protein coupling (Tateyama and Kubo, 2007; 2008; Xu et al., 2007). Four 4 basic amino acid residues (RRKK) appear to be inhibitory for $G\alpha_{q/11}$ coupling unless masked by the long C-tail in the $mGlu_{1a}$ splice variant (Mary et al., 1998; Tateyama and Kubo, 2008). Still, even the shorter splice variants retain the ability to activate $G\alpha_{q/11}$, but do so more slowly and with a right-shifted dose–response (Mary et al., 1998; Tateyama and Kubo, 2008). Finally, the exposed RRKK motif also abolishes coupling to $G\alpha_{i/o}$ (Tateyama and Kubo, 2008).

Nevertheless, questions remain about the role of the C-tail in $mGlu_1$ receptor G protein coupling. First, does the $mGlu_1$ receptor C-tail act as an activator of G proteins, or simply regulate activation that primarily resides elsewhere in the receptor, such as the second and third intracellular loops (Francesconi and Duvoisin, 1998)? Second, is the RRKK motif the major regulator of G protein coupling in the $mGlu_1$ receptor C-tail, or are other motifs important as well?

To begin to address these questions, a C-terminally truncated $mGlu_1$ receptor mutant was constructed and dual coupling ($G\alpha_{q/11}$ and $G\alpha_{i/o}$) was simultaneously examined by measuring two forms of N-type calcium channel modulation in sympathetic neurons from the rat superior cervical ganglion. These pathways include a pertussis toxin (PTX)-sensitive, $G\beta\gamma$-mediated pathway that shows characteristic voltage dependence, and a $G\alpha_{q/11}$-mediated, voltage independent pathway (Kammermeier and Ikeda, 1999).

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2. Materials and methods

2.1. Cell isolation, cDNA injection and preparation

A detailed description of the cell isolation and cDNA injection protocol is published elsewhere (Ikeda, 1997). The animal protocols used were approved by the University Committee on Animal Resources (UCAR). Briefly, both superior cervical ganglia were removed from adult male Wistar rats (150–300 g) following decapitation, and incubated in Earle's balanced salt solution (Life Technologies Inc., Rockville, MD) containing 0.6 mg/ml trypsin (Worthington Biochemicals, Freehold, NJ), and 0.8 mg/ml collagenase D (Boehringer Mannheim Biochemicals, Indianapolis, IN) for 1 h at 35 °C. Cells were then centrifuged (50×*g*), transferred to minimum essential medium (Fisher Scientific, Pittsburgh, PA), plated on poly-L-lysine coated 35 mm polystyrene tissue culture dishes and incubated (95% air and 5% CO_2; 100% humidity) at 37 °C prior to DNA injection. After injection, cells were incubated overnight at 37 °C and imaging or patch clamp experiments were performed the following day.

Injection of cDNA was performed with an Eppendorf FemtoJet microinjector and Injectman NI2 micromanipulator (Brinkmann, Westbury, NY) 4–6 h following cell isolation. Plasmids are stored at −20 °C as a 1 μg/μl stock solution in TE buffer (10 mM TRIS, 1 mM EDTA, pH 8). Neurons were coinjected with "enhanced" green fluorescent protein cDNA (0.005 μg/μl; $pEGFPN_1$) or pdSRec-nuc (Clontech Laboratories) to facilitate later identification of successfully injected cells. All constructs were sequence verified prior to use in experiments. PCR products were purified with Qiagen (Valencia, CA) silica membrane spin columns or Sigma GenElute Plasmid Midiprep Kits prior to restriction digestion and ligation. Plasmids were propagated in Top10 bacteria (InVitrogen) and midipreps prepared using Qiagen anion exchange columns.

The $mGlu_1$-dCT (pCDNA3.1+) construct and the analogous myc-tagged construct was generated by PCR from the original full-length $mGlu_1$ construct (pCDNA3.1; generously provided by Stephen R. Ikeda, NIAAA, Rockville, MD), using the following primers: 5′-gatacactcgagaccaccatggtccggctcctcttgattttc, and 5′-ctgcgctctagactaggcaatgatgatgtacatc, resulting in a 2520 base pair product corresponding to the coding sequence of amino acids 1–840 of the rat $mGlu_1$ receptor sequence, plus a stop codon. This sequence was re-ligated into pCDNA3.1+ at XbaI/XhoI cut sites.

2.2. Electrophysiology and data analysis

Patch clamp recordings were made from 8250 glass (Garner Glass, Claremont, CA). Pipette resistances were generally 1–3 MΩ, yielding uncompensated series resistances of 2–7 MΩ. Series resistance compensation of 80% was used in all recordings. Data were recorded using an EPC-7 patch clamp amplifier from HEKA Elektronik (Germany). Voltage protocol generation and data acquisition were performed using custom data acquisition software (donated by Stephen R. Ikeda, NIAAA, Rockville, MD) on a Macintosh G4 computer with an Instrutech ITC16 data acquisition board (HEKA). Currents were sampled at 0.5–5 kHz low-pass filtered at 3 kHz using the filter in the patch clamp amplifier, digitized, and stored on the computer for later analysis. All experiments were performed at 21–24 °C (room temperature). Data analysis was performed using Igor Pro software (WaveMetrics, Lake Oswego, OR).

For calcium current recordings, the external (bath) solution contained (in mM): 145 tetraethylammonium (TEA) methanesulfonate (MS), 10 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 15 glucose, 10 $CaCl_2$, and 300 nM tetrodotoxin, pH 7.4, and osmolality of 320 mOsm/kg. The internal (pipette) solution contained: 120 N-methyl-D-glucamine (NMG) MS, 20 TEA, 11 EGTA, 10 HEPES, 10 sucrose, 1 $CaCl_2$, 4 MgATP, 0.3 Na_2GTP, and 14 tris creatine phosphate, pH 7.2, and osmolality of 300 mOsm/kg. For M-current recordings, the external solution contained: 150 NaCl, 2.5 KCl, 10 HEPES, 1 $MgCl_2$, 2 $CaCl_2$, 15 glucose, and 300 nM tetrodotoxin, pH 7.4, and osmolality of 320 mOsm/kg. The internal solution contained: 150 KCl, 0.1 K_4BAPTA, 10 HEPES, 4 MgATP, and 0.1 Na_2GTP, pH 7.2, and osmolality of 300 mOsm/kg. The glutamate concentration in all experiments was 100 μM. The glutamate concentration used was 100 μM.

2.3. Immunofluorescence

Superior cervical ganglion neurons were plated on glass cover slips and injected with cDNA coding for either myc-$mGlu_1$ or myc-$mGlu_1$-dCT and with nuclear targeted dSRed to identify injected cells. Cells were exposed to 9 μg/ml of primary monoclonal anti-myc antibody with a FITC conjugate (Sigma) for 25 min, then washed in phosphate buffered saline. Confocal images were then taken of labeled neurons within 30 min using a Nikon Eclipse Ti scanning confocal microscope. FITC and dSRed images were taken using 488/530 nm and 543/585 nm (excitation/emission wavelength), respectively. Image analysis was done using Igor Pro software (Wavemetrics).

3. Results

3.1. mGlu1 receptor; C-terminal deletion

Several recent studies have implicated the C-terminal tail of mGlu receptors in the G protein coupling process (Mary et al., 1998; Tateyama and Kubo, 2007; 2008; Xu et al., 2007). To determine the specific role of the intracellular C-terminus of $mGlu_1$ receptor in G protein coupling, a mutant receptor was constructed with the C-terminus deleted. This receptor, termed $mGlu_1$-dCT, consists of the first 840 amino acids of the rat $mGlu_1$ receptor protein. The predicted topology of the resulting receptor has seven transmembrane domains but lacks any intracellular C-terminus present to various degrees in several $mGlu_1$ receptor splice variants.

To evaluate signaling, the wild type or dCT mutant of $mGlu_1$ was expressed in isolated sympathetic neurons from the rat superior cervical ganglion. The primary calcium channels natively expressed in these neurons are N-type ($CaV_{2.2}$) channels, which can be inhibited by two primary pathways: a voltage dependent, Gβγ-mediated pathway (Herlitze et al., 1996; Ikeda, 1996) usually associated with activation of pertussis toxin (PTX)-sensitive G proteins (Hille, 1994), and a $G\alpha_{q/11}$-mediated pathway that likely involves PIP_2 depletion upon phospholipase C activation (Gamper et al., 2004; Kammermeier and Ikeda, 1999). Conveniently, when assaying receptor activation using whole-cell patch clamp to measure calcium current inhibition in superior cervical ganglion neurons, these pathways can be distinguished by their voltage dependence (Kammermeier and Ikeda, 1999). The Gβγ-inhibition is strongest at moderate voltages and can be strongly reversed for a few tens of milliseconds following a depolarizing step to +80 mV (Bean, 1989; Elmslie et al., 1990). By contrast, the $G\alpha_{q/11}$-inhibition is similar at all voltages and is unaltered by depolarization. Thus, by closely examining the magnitude and voltage dependence of current inhibition, this system can provide an accurate, real-time measurement of the degree of $G\alpha_{i/o}$ and $G\alpha_{q/11}$ protein activation by the receptor, respectively.

Fig. 1 illustrates glutamate concentration–response curves for wild type $mGlu_1$ and $mGlu_1$-dCT using superior cervical ganglion calcium current inhibition as the assay for receptor activity. The wild type receptor exhibited a strong maximal inhibition of around 60% and an EC_{50} of 1.3 μM (Fig. 1A, *upper* and B). By contrast, $mGlu_1$-dCT showed a weaker overall efficacy with a maximal inhibition of just over 20%. However at 3.5 μM, the EC_{50} for the mutant receptor was similar to the wild type (Fig. 1A, *lower* and B).

3.2. Voltage dependence of calcium current modulation

Closer examination of revealed that the calcium current inhibition induced by $mGlu_1$-dCT was not only smaller in magnitude than that