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The intra-molecular activation mechanisms of the dimeric metabotropic glutamate receptor 1 differ depending on the type of G proteins

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ABSTRACT

Metabotropic glutamate receptor 1 (mGlu1) functions as a homodimer and activates not only the Gq but also the Gi/o and Gs pathways. Because of the dimeric configuration, different pathways could be activated either through the glutamate-bound subunit (cis-activation) and/or the other one (trans-activation). We here examined whether the intra-molecular activation mechanisms in the mGlu1 differ depending on the type of coupling G proteins, using various combinations of mGlu1 constructs that lack glutamate binding and/or G-protein coupling. The cis- or trans-activation alone was confirmed to trigger the Gq-coupled intracellular Ca^{2+} transient. In contrast, the Gi/o-coupled G protein-dependent inward rectifying potassium (GIRK) channels were not activated either through the cis- or trans-activation alone. When one subunit of dimeric mGlu1 lacked the G-protein coupling, a significant decrease in the glutamate-induced GIRK current density was also observed. As the G protein-coupling-deficient subunit did not decrease the cell surface expression of mGlu1 and the Gq-coupled Ca^{2+} transient, it was suggested that the coupling deficiency in one subunit of mGlu1 attenuates the Gi/o but not Gq coupling. In conclusion, multiple G-protein signaling was differentially activated by different intra-molecular activation mechanisms of the dimeric mGlu1.

1. Introduction

Metabotropic glutamate receptors (mGlu) are the key molecules for neuronal transmission and signaling (Nicoletti et al., 2010). mGlu are G protein-coupled receptors (GPCRs) that belong to family C, to which γ-aminobutyric acid type B ($GABA_B$) receptor also belongs. The family C GPCRs are dimers and have a large extracellular domain (ECD) where the ligand recognition sites locate (Nicoletti et al., 2010; Pin et al., 2003). The dimeric and/or oligomeric configuration has also been also reported in family A GPCRs, but the formation of oligomers is not necessary for their signaling (Park et al., 2004). In contrast, the dimeric configuration is requisite for family C GPCRs to function as the signaling molecules (Pin et al., 2003). The ligand binding that occurs at the ECD should be transmitted to their 7 trans-membrane domain (7TMD) for activation of the G proteins. X-ray crystallographic analyses of mGlu have revealed that a glutamate stabilizes the closed conformation of one ECD, which has been suggested to cause dimeric rearrangement of the ECDs (Kunishima et al., 2000; Muto et al., 2007). The dimeric rearrangement was demonstrated in the intracellular domains (ICDs) (Tateyama et al., 2004), with some conformational changes of the 7TMD (Brock et al., 2007; Yamashita et al., 2008). The ligand-induced dimeric rearrangement has also been demonstrated in $GABA_B$ receptors (Matsushita et al., 2010), suggesting that the dimeric rearrangement is an important mechanism for the family C GPCRs to activate G proteins. Because of the dimeric configuration of the family C GPCR, G proteins might be activated via the ligand-bound subunit (cis-activation) and/or the other one (trans-activation). Alternatively, ligand binding on two subunits might be requisite for the G-protein activation. In the case of homodimeric mGlu5, the Gq pathway can be triggered either through the cis- or trans-activation alone (Brock et al., 2007). In addition, the heterodimeric $GABA_B$ receptor is accepted to activate the Gi/o proteins through trans-activation, since the receptor

Abbreviations: mGlu, metabotropic glutamate receptor; GPCR, G protein-coupled receptor; $GABA_B$, γ-aminobutyric acid type B; ECD, extracellular domain; 7TMD, 7 trans-membrane domain; ICD, intracellular domain; GIRK, G protein-dependent inward rectifying potassium; TRPC3, transient receptor potential canonical 3; C1, C-terminal tail of GB1; C2KKTN, C-terminal tail of GB2 with KKTN; YFP, yellow fluorescent protein; HEK293T, human embryonic kidney 293T; CHO, Chinese hamster ovary; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; $\Delta[Ca^{2+}]_i$, maximal increase in the intracellular Ca^{2+} concentration; FRET, fluorescent resonance energy transfer; $[cAMP]_i$, intracellular cAMP levels; PTX, pertussis toxin; [glutamate], glutamate concentration; A.U., arbitrary units; WT, wild type; FL-WT-C2, FLAG-tagged WT-C2KKTN.

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consists of a ligand-sensing subunit of GB1 and a Gi/o coupling subunit of GB2 (Galvez et al., 2001; Margeta-Mitrovic et al., 2001).

A subtype of mGlu, mGlu1a, is known to activate not only the Gq pathway but also the Gs and Gi/o pathways, which may be responsible for the diverse responses in neuronal cells (Kammermeier, 2009; Kitano et al., 2003; Sugiyama et al., 2008). The multiple signaling via mGlu1a is dependent on the mGlu1a expression system (Selkirk et al., 2001) and differentially affected by mutations or phosphorylation of the ICD of mGlu1a (Francesconi and Duvoisin, 1998, 2000). In addition, a difference in the activated conformation of mGlu1a has been suggested to alter the coupling profile of mGlu1 (Sheffler and Conn, 2008; Tateyama and Kubo, 2006). From these studies, it could be assumed that the difference in the intramolecular activation mechanisms, such as cis- or trans-activation, might differentially affect the Gs or Gi/o coupling of mGlu1a. In the present study, we confirmed that the Gq pathway via mGlu1 was activated either through the cis- or the trans-activation alone (Brock et al., 2007) and newly found that the Gi/o pathway was not triggered either through the cis- or the trans-activation alone. Similarly, the Gs pathway was not triggered through the trans-activation alone. We further investigated the effects of glutamate-binding or G protein-coupling deficiency in one subunit of mGlu1 on the multiple signaling.

2. Materials and methods

2.1. Constructs and expression system

Point mutants of rat mGlu1a, mGlu2 and G protein-dependent inward rectifying potassium type 2 (GIRK2) channel were constructed by PCR with mutated primers and KOD plus ver. 2 Taq polymerase (Toyobo, Osaka, Japan). Transient receptor potential canonical type 3 (TRPC3) channel was isolated from a rat brain cDNA library by PCR. Fragments of the C-terminal tail of GB1 (C1) or GB2 with a retention signal KKTN (C2KKTN) were amplified by PCR with designed primer sets and then inserted after Arg857 using a SphI site of the mGlu1a constructs (Brock et al., 2007). The FLAG epitope was inserted into the N-terminal region of mGlu1-C2KKTN after the signal peptide sequence, as previously reported (Abe et al., 2003). The coding sequence of yellow fluorescent protein (YFP) was inserted between Ile685 and Leu686 at the 2nd intracellular loop of mGlu1-C1, i2-YFP-C1, by blunt-end ligation (Tateyama et al., 2004). DNA sequences of the constructs were confirmed and then subcloned into pCXN2 expression vector except for S196A-GIRK2, a mutant GIRK2 which is resistant to the inhibition by protein kinase C-dependent phosphorylation (Mao et al., 2004). The S196A-GIRK2 was subcloned into pIRES2-GFP (Clontech, Mountain View, CA, USA). Human embryonic kidney 293T (HEK293T) and Chinese hamster ovary (CHO) cells were transfected with the plasmid DNA using Lipofectamine2000 (Invitrogen, Carlsbad, CA, USA) and seeded onto cover glasses. Experiments using HEK293T cells were carried out 24–48 h after transfection and those using CHO cells were done after 48–72 h. Before electrophysiological experiments or imaging, cells were incubated for more than 30 min in Hank's balanced salt solution (Invitrogen) supplemented with 1 mM Ca^{2+} and 0.3 mM Mg^{2+} at room temperature.

2.2. Monitoring of the changes in $[Ca^{2+}]_i$ and $[cAMP]_i$

Changes in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in HEK293T cells were monitored every 3 s by using fura2-AM (Invitrogen), and the ratio of the fluorescence intensity (excitation 340 nm/380 nm, emission 535 nm) was calculated. Cells loaded with the fura2-AM were placed in a recording chamber filled with 250 μL of the bath solution containing (in mM) 140 NaCl, 1 $CaCl_2$, 4 KCl, 0.3 $MgCl_2$, and 10 HEPES (pH adjusted to 7.4 with NaOH). 250 μL of twice-concentrated glutamate stock solved in the bath solution was applied to the recording chamber by pipetting. Peak amplitudes of the ratio after glutamate application were obtained from cells expressing YFP, a transfection marker, and the maximal increase in the $[Ca^{2+}]_i$ ($\Delta[Ca^{2+}]_i$) was calculated by subtraction of the basal amplitude measured 0–30 s before glutamate application.

To evaluate the Gs and Gq coupling of mGlu1 constructs in CHO cells, we simultaneously monitored the efficiency of fluorescence resonance energy transfer (FRET) of ICUE2, a probe for cAMP concentration (DiPilato et al., 2004; Dunn et al., 2006) and the emission ratio of indo-1 (excitation 340 nm, emission 405 nm/470 nm; Invitrogen) every 3 s. The intracellular cAMP concentration ($[cAMP]_i$) was evaluated as the reversed ratio of FRET (excitation 436 nm, emission 470 nm/535 nm) and then the reversed ratios were normalized to the basal values (averaged value for 30 s before glutamate application). Cells that showed either $\Delta[Ca^{2+}]_i > 0.05$ or $[cAMP]_i > 1.05$ were defined as glutamate-responsive cells for the analyses.

2.3. Electrophysiology

The Gi/o coupling of mGlu constructs was evaluated as the amplitude of the glutamate-induced inward currents through the GIRK2 channels. The Gq coupling of mGlu1 constructs was evaluated as the amplitude of the glutamate-induced inward current through the La^{3+} sensitive TRPC3 channels (Hartmann et al., 2008). Macroscopic currents were recorded by the whole cell patch clamp technique at room temperature (Tateyama and Kubo, 2008). The internal solution for recording of GIRK currents contained (in mM) 120 KCl, 5 K_2-ATP, 10 NaCl, 3 EGTA, 10 HEPES, 0.1 $CaCl_2$, 4 $MgCl_2$, and 0.3 μM GTP (pH adjusted to 7.4 with KOH). The internal solution for recording of TRPC3 currents contained (in mM) 140 KCl, 4 Na_2-ATP, 0.3 EGTA, 10 HEPES, and 5 $MgCl_2$ (pH adjusted to 7.4 with KOH). The composition of the bath solution was the same as that for imaging. 140 mM Na^+ ions were replaced with 140 mM K^+ ions for recording of GIRK currents and the Ca^{2+} ions were replaced with Ba^{2+} ions for recording of TRPC3 currents. After the glutamate-induced current reached a maximum, the mean amplitudes for 0.5 s were measured at a holding potential of −80 mV. The current amplitude induced by 1 mM glutamate was normalized by the cell capacitance to calculate the current density. To evaluate the glutamate sensitivity of the responses, the current amplitude evoked by various concentrations of glutamate ([glutamate]) was normalized to that evoked by 1 mM glutamate in each cell. The relationship between [glutamate] and GIRK current was fitted to a logistic curve (Origin8; OriginLab, Northampton, MA, USA). The voltage-dependence of the glutamate-induced current was evaluated by applying a ramp protocol, −120 mV to 40 mV at a rate of 0.4 mV/ms, at 0.2 Hz. The current traces shown in figures were off-line smoothed by averaging 10 adjacent points (Origin 8) to clearly demonstrate the glutamate-induced changes.

2.4. Immunocytochemistry

HEK293T cells were transfected with the same amount of cDNAs (1 μg) of mGlu1 constructs and S196A-GIRK2. Twenty-four hours after transfection, the immunocytochemical experiments were carried out, as previously reported (Tateyama and Kubo, 2008). The cells were fixed with 2% paraformaldehyde for 2 min at 4 °C without Triton-X, which permeabilizes membrane, and then incubated with antibodies (1st antibody, FLAG-antibody clone M2, 4 μg/ml, Sigma–Aldrich, Tokyo, Japan; 2nd antibody, Alexa-564-conjugated anti-mouse IgG, Invitrogen). Fluorescence images were captured with a BX-50 microscope (Olympus, Tokyo, Japan) equipped with an AxioCam CCD camera unit (Carl Zeiss, Jena, Germany) and analyzed with Aquacosmos software (Hamamatsu Photonics, Hamamatsu, Japan). To exclude fluorescence signals of Alexa-564 from the intracellular compartment, we first selected cells expressing GFP (excitation at 470–495 nm and emission at 510–550 nm, more than 6 arbitrary units (A.U.)) to determine the region for measurement. The fluorescence intensity of Alexa-564 (excitation at 520–550 nm and emission more than 580 nm) was measured at each pixel within the selected region. The intensity was normalized by the area to compare the surface expression level of mGlu1 constructs in each group.

2.5. Analysis and statistics

All data are expressed as the means ± S.E., with n indicating the number of data. A statistical significance was estimated by unpaired Student's t-test or Dunnet's t-test; values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. Trans-activation alone triggers Gq but not Gi/o or Gs

We first confirmed that the Gq pathway could be triggered through the trans-activation of mGlu1 alone, by using glutamate-binding-deficient R78L- and G protein-coupling-deficient F781S-mGlu1a (Francesconi and Duvoisin, 1998; Jensen et al., 2000) (Fig. 1A). Homo-multimers of R78L and F781S could not increase the $[Ca^{2+}]_i$ upon 1 mM glutamate application, although they were well expressed on the plasma membrane (Fig. S1). In contrast, the hetero-multimer formed by co-expression of R78L- and F781S-mGlu1a did increase the $[Ca^{2+}]_i$ (Fig. 1B and D). These results indicated that the Gq pathway can be triggered through the trans-activation alone, because only the trans-activation pathway is available in the combination of R78L and F781S (Fig. 1A). Then the Gi/o coupling of the hetero-multimer was investigated by recording inward currents through GIRK2 channels in HEK293T cells, since mGlu1a activates the Pertussis toxin (PTX)-sensitive Gi/o pathway in HEK293T cells (Kitano et al., 2003; Tateyama and Kubo, 2008). Activation of wild-type (WT) mGlu1a increased the