Role of the Large Extracellular Domain of Metabotropic Glutamate Receptors in Agonist Selectivity Determination*

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Metabotropic glutamate receptors consist of at least six different subtypes termed mGluR1–mGluR6. They belong to the family of G protein-coupled receptors and commonly possess an unusually large extracellular domain preceding the seven transmembrane segments. mGluR1 and mGluR2 show similar affinities for L-glutamate, quisqualate, and tACPD in Xenopus oocytes. Replacement of the extracellular domain up to about one-half of the amino-terminal extracellular domain of mGluR1 with the corresponding portion of mGluR2 generated a pattern of the agonist selectivity characteristic of mGluR2. The acquisition of this property in agonist selectivity was further indicated by the selective responses of these chimeric receptors to an mGluR2-specific agonist, (2S,1'R,2'R,3'R)-2-(2,3-dicarboxycyclopropyl)glycine. This investigation demonstrates that the extracellular domain of mGluR is critical in determining agonist selectivity and that the mode of determination of agonist selectivity of mGluR is different from that of other G protein-coupled receptors for small molecule transmitters.

Glutamate neurotransmission plays an important role in neuronal plasticity and neurotoxicity in the central nervous system (Choi and Rothman, 1990; Bliss and Collingridge, 1993). The diverse functions of glutamate neurotransmission are mediated by a variety of glutamate receptors that can be classified into two distinct groups termed ionotropic and metabotropic receptors (mGluRs) on the basis of electrophysiological, pharmacological, and molecular studies (Nakanishi, 1992). Recent molecular studies have indicated that mGluRs consist of at least six different subtypes termed mGluR1–mGluR6 (Masu et al., 1991; Houamed et al., 1991; Tanabe et al., 1992; Abe et al., 1992; Nakajima et al., 1993). The six mGluRs show a high degree of sequence homology within this receptor family but share no sequence similarity to conventional G protein-coupled receptors and possess a large extracellular domain preceding the seven putative transmembrane segments (Masu et al., 1991; Nakanishi, 1992). Thus, mGluRs form a novel family of G protein-coupled receptors. The six mGluR subtypes can be subdivided into three subgroups according to their sequence homology, signal transduction, and agonist selectivities (Nakanishi, 1992). mGluR1 and mGluR5 are coupled to inositol trisphosphates (IP3)/Ca<sup>2+</sup> signal transduction and show a strong agonist selectivity to quisqualate (Masu et al., 1991; Aramori and Nakanishi, 1992; Abe et al., 1992). The other four mGluR subtypes are linked to the inhibitory cyclic AMP cascade, but the agonist selectivities of mGluR2/mGluR3 and mGluR4/mGluR6 are totally different from each other. The former subtypes potently react with trans-1-amino-1,3-cyclopentanedicarboxylate (tACPD), whereas the latter subtypes effectively interact with L-2-amino-4-phosphonobutyrate (Tanabe et al., 1992; Tanabe et al., 1993; Nakajima et al., 1993).

Although the physiological roles of the mGluR family largely remain to be clarified, the individual mGluR subtypes seem to have their own functions in glutamate neurotransmission in a variety of neuronal cells (Nakanishi, 1992; Schoepp and Conn, 1993; Nakajima et al., 1993).

G protein-coupled receptors interacting with small molecule ligands such as adrenaline and acetylcholine possess small extracellular amino-terminal domains and interact with the corresponding ligands in their transmembrane pockets (Frielle et al., 1988; Koblika et al., 1988; Kubo et al., 1988). In contrast, the binding of large glycohormones such as thyrotropin and lutropin-choriogonadotropin occurs by interaction with the large extracellular domains of these glycomethylene receptors (Xie et al., 1990; Nagayama et al., 1991). mGluRs differ in their structural characteristics from either type of the G protein-coupled receptors. These receptors, though interacting with the small molecule glutamate, possess an unusually large extracellular amino-terminal domain consisting of 550–570 amino acid residues. This structural feature raises an interesting question concerning the ligand-binding domains of the mGluR family. To address this question, we designed the utilization of the difference in the agonist selectivity and signal transduction between mGluR1 and mGluR2. We constructed a series of chimeric receptors between these two receptor subtypes and determined the agonist selectivities of the resultant chimeric receptors expressed in Xenopus oocytes. The results presented here indicate that the extracellular domain preceding the seven transmembrane segments...
of mGlR serves as a determinant that confers the agonist selectivity of this receptor family.

**EXPERIMENTAL PROCEDURES**

**Materials**—In vitro mutagenesis kit was purchased from Bio-Rad. The cDNA for the bovine homologue of G_{i,6} (Strathmann and Simon, 1990; Nakamura et al., 1991) and (2S,1'R,2'R,3'R)-2-(2,3-dicarboxycyclopropyl) glycine (previously referred to as DCG-1/4 and renamed DCG-IV) were kindly provided by Drs. T. Nukada (University of Tokyo) and Y. Ohhune (Suntory Institute, respectively). Other reagents were obtained as described (Masu et al., 1991; Hayashi et al., 1992; Abe et al., 1992). cDNAs for rat mGlR1α and mGlR2 were prepared as reported previously (Masu et al., 1991; Tanabe et al., 1992).

**Construction of Chimeric Receptor cDNAs**—Six restriction sites (ClaI, NdeI, AfIII, MluI, BglII and EcoRV; see Fig. 1) at equivalent positions in the cDNAs covering the extracellular amino-terminal domains of rat mGlR1 (Masu et al., 1991) and mGlR2 (Tanabe et al., 1992) were used to construct chimeric receptor cDNAs; the NdeI site preexisted in the mGlR2 cDNA, while the others were introduced by site-directed mutagenesis (Kunkel, 1987; Kakizuka et al., 1990; Kozak et al., 1990) using the in vitro mutagenesis kit. These sites were chosen because they are unique in both cDNA sequences covering their extracellular domains and the chimeric formations cause no insertion, deletion, or substitution in the amino acid sequences of mGlR1 and mGlR2. The authenticity of the various chimeric receptor cDNAs constructed was confirmed by sequence determination in combination with restriction enzyme analysis.

**Electrophysiological Measurements of Chimeric Receptors in Xenopus Oocytes**—Preparations of oocytes and in vitro cRNA synthesis were carried out as described previously (Masu et al., 1991). Electrophysiological measurements were performed by two microelectrode techniques under voltage clamp at -60 mV. To potentiate the electrophysiological response in Xenopus oocytes, the cRNA for the bovine homologue of G_{i,6} (Strathmann and Simon, 1990; Nakamura et al., 1991) was co-injected with each chimeric receptor cRNA (20 ng) (Nakamura et al., 1992). The oocytes were incubated at 19°C for 2-6 days. For electrophysiological measurements, oocytes were perfused by a constant stream of a standard solution (95 mM NaCl, 2 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 5 mM HEPES pH 7.5), and drugs tested were applied by switching the flow, except that bath application was used to make high concentrations of quisqualate (≥1 mM), tACPD (≥1 mM), and 100 μM DCG-IV; these chemicals were adjusted to neutral pH before bath application. Dose-response curves for L-glutamate, quisqualate, and tACPD were determined by measuring peak currents after serial application of various concentrations of these compounds.

**Statistical Analysis**—Theoretical curves for determination of ED₅₀ were drawn according to the equation \(E = E_{\text{max}}/(1 + A^{n})\), where \(E\) represents the current response; \(E_{\text{max}}\), the maximum response (100% of the respective agonist; \(A\), the concentration of agonist; \(n\), the Hill coefficient).

**RESULTS**

**Construction and Expression of Chimeric Receptors**—Our previous studies indicated that mGlR1 expressed in Xenopus oocytes is capable of inducing electrophysiological responses to ligand application through the activation of the oocyte IP₃/Ca⁺⁺ signal transduction (Masu et al., 1991). In contrast, no such response was evoked for mGlR2 in Xenopus oocytes, because this receptor subtype is linked to the inhibitory cyclic AMP cascade (Tanabe et al., 1992). The two receptor subtypes also showed distinct properties in their agonist selectivities. mGlR1 responds effectively to quisqualate but less so to tACPD, whereas mGlR2 responds potently to tACPD but not appreciably with quisqualate (Masu et al., 1991; Tanabe et al., 1992; Aramori and Nakamichi, 1992). To investigate whether the extracellular amino-terminal domain of mGlR is involved in determining the agonist selectivity of mGlR, we first constructed a chimeric receptor in which the extracellular domain of mGlR1 was almost entirely replaced with the corresponding region of mGlR2 (TQ6 in Fig. 1). Because this chimeric receptor retained the cytoplasmic region that is responsible for coupling to IP₃/Ca⁺⁺ signal transduction, it was expected that the TQ6 receptor is capable of inducing an electrophysiological response to L-glutamate application when expressed in Xenopus oocytes. Our preliminary experiment indicated that TQ6 not only responded to L-glutamate but also, like mGlR2, showed a more preferred response to tACPD than quisqualate. We thus constructed a series of mGlR chimeras between mGlR1 and mGlR2 by sequentially replacing cDNA restriction fragments at the corresponding restriction sites situated in the extracellular domains of these two mGlR subtypes. A total of 11 chimeric receptors thus constructed consisted of two series of reciprocal receptors, termed TQ and QT series, as illustrated in Fig. 1. In the TQ series, the extracellular amino-terminal sequences of mGlR1 were substituted for the homologous regions of mGlR2 in the direction from the amino terminus to the carboxyl terminus. Chimeras in the QT series were mirror images of those in the TQ series in that the extracellular sequences of mGlR1 were replaced with the corresponding regions of mGlR2 in the reverse direction. The cRNAs for the native and chimeric receptors were synthesized in vitro and were injected into Xenopus oocytes to test for electrophysiological responses to L-glutamate or various agonists. We co-injected the cRNA for a bovine G protein, G_{i,6}, (Strathmann and Simon, 1990; Nakamura et al., 1991), that was reported to potentiate electrophysiological responses (Nakamura et al., 1992) and thus yielded more reliable data. We also confirmed that oocytes injected with distilled water or with mGlR2 mRNA showed no oscillatory responses resulting from the activation of oocyte IP₃/Ca⁺⁺ signal transduction after application of L-glutamate or other agonists.

**Measurements of Electrophysiological Responses**—mGlR1 evoked a maximal electrophysiological response to application of 100 μM L-glutamate when expressed in Xenopus oocytes.
(Masu et al., 1991). However, in some chimeric formations, no obvious or very little electrophysiological responses were induced by application of 100 μM L-glutamate. To address whether various chimeric receptors were functionally expressed in Xenopus oocytes, we first measured the electrophysiological responses of the individual chimeric receptors after the addition of high concentrations (10 mM each) of L-glutamate, tACPD, and quisqualate. The result of this analysis is presented in Fig. 2, and several characteristic features of a series of chimeric receptors are summarized as follows.

All but TQ1 in the TQ series of chimeric receptors and only QT5 among the QT series of receptors showed appreciable electrophysiological currents in response to L-glutamate application. No other chimeric receptors (TQ1 and QT1-QT4) showed measurable responses to any of the three agonists. The failure of these receptors in responding to a high concentration of L-glutamate may be due to insufficient synthesis, inappropriate membrane incorporation, or some molecular incompatibility to maintain L-glutamate binding. These mechanisms, however, remain to be clarified. Among the positively responding chimeric receptors, TQ6 evoked a response that was comparable with that of mGluR1, whereas the five other chimeric receptors were reduced in their maximal responses to application of L-glutamate by about one-third that of the native mGluR1.

At a high concentration of quisqualate and tACPD, the native mGluR1 responded efficiently to quisqualate and less so with tACPD as compared with L-glutamate. QT5, which retains a large extracellular portion of mGluR1, responded equally well to quisqualate but lost the ability to interact with tACPD. In contrast, the reactivity with tACPD and quisqualate was reversed in the TQ3-TQ6 chimeric receptors, and these receptors responded relatively well to tACPD but failed to interact with quisqualate. TQ2 differed from either type of the above chimeric receptors and showed the potency to interact with all three agonists to a similar extent. These results strongly suggest that the extracellular domain preceding the seven transmembrane segments of mGluR is responsible for determining the agonist selectivity of this receptor.

**Dose-Response Analyses**—To confirm the above findings, we determined dose-response curves of the positively responding chimeric receptors by applying various concentrations of L-glutamate, quisqualate, and tACPD. The results are presented in Fig. 3 and Table I and can be summarized as follows.

The native mGluR1 showed a rank order of agonist potency with quisqualate > L-glutamate > tACPD as reported previously (Masu et al., 1991). Half-maximal effective doses (ED50) of the respective agonists were 7 × 10^(-8), 1 × 10^(-5), and 2.4 × 10^(-4) M. All chimeric receptors examined were reduced in their ED50 values for L-glutamate by one order or more of magnitude when compared with the native mGluR1. However, in QT5 where a large extracellular domain is composed of the mGluR1 structure, the characteristics of mGluR1 of interacting with quisqualate more efficiently than with L-glutamate was maintained, although the reactivity with tACPD, unlike mGluR1, was totally lost in this chimeric formation.

In a series of the TQ1-TQ6 chimeric receptors, quisqualate was found to be inactive in inducing electrophysiological responses to serial application of indicated concentrations of L-glutamate, quisqualate, and tACPD. The responses were given as percentages of that elicited with 10 μM of the respective agonist. Data represent the means of the responses obtained from at least three experiments. For other explanations, see Fig. 2.

**Table I**

| Receptor | L-Glutamate | tACPD | Quisqualate |
|----------|-------------|-------|-------------|
| TQ2      | 520 ± 210 (n = 5) | 1200 ± 190 (n = 3) | 650 ± 240 (n = 4) |
| TQ3      | 160 ± 35 (n = 3) | 1300 ± 700 (n = 4) | (-)          |
| TQ4      | 160 ± 39 (n = 5) | 650 ± 210 (n = 4) | (-)          |
| TQ5      | 110 ± 12 (n = 4) | 870 ± 29 (n = 4) | (-)          |
| TQ6      | 100 ± 12 (n = 4) | 110 ± 8.5 (n = 3) | t(-)         |
| QT5      | 180 ± 65 (n = 4) | (-)          | 30 ± 22 (n = 3) |

mGluR1 10 ± 1.8 (n = 4) 240 ± 120 (n = 6) 0.70 ± 0.39 (n = 3)
response up to the concentration of 10 mM. In contrast, tACPD efficiently evoked responses in these chimeric receptors. Furthermore, in TQ6 in which the extracellular domain of mGluR1 was almost entirely replaced with the corresponding domain of mGluR2, the agonist potencies of tACPD and L-glutamate were virtually identical with each other. This agonist selectivity of TQ6 was very similar to that reported for the native mGluR2 as assessed by measuring agonist-mediated inhibition of forskolin-stimulated cyclic AMP formation in mGluR2-expressing Chinese hamster ovary cells (Tanabe et al., 1992).

TQ2 responded to all three agonists comparably but less efficiently. This receptor thus differed from any of the above chimeric receptors and seemed to represent an intermediate form between the QT5 type and the TQ3-TQ6 type of chimeric receptors in terms of its agonist selectivity.

The above results, taken altogether, strongly indicated that about one-half of the amino-terminal extracellular domain (up to site A) is critical in determining the agonist selectivity of mGluR.

Responsiveness to Dicarboxycyclopropyl Glycine—In the above experiments, a distinct pattern in responsiveness to quisqualate and tACPD allowed the assignment of a structural determinant of the agonist selectivity at the extracellular amino-terminal domain of mGluR. However, the above result also showed that the native mGluR1 is capable of reacting with tACPD comparably with or more effectively than the TQ3-TQ6 chimeric receptors (Table I). It thus remained possible, though very unlikely, that the TQ3-TQ6 chimeric receptors could maintain a fundamental property characteristic of mGluR1 but could selectively lose a binding site specific for quisqualate. To exclude this possibility, we designed different experiments with the aid of a new glutamate analogue DCG-IV. DCG-IV was developed by Ohfune et al. (1993), who modified the 3' carbon of 2-(carboxycyclopropyl)glycine that was identified as a selective agonist for the mGluR family (Hayashi et al., 1992). An agonist selectivity of DCG-IV was analyzed by examining its effect on signal transduction of different mGluR subtypes expressed in Chinese hamster ovary cells, and this analysis indicated that DCG-IV is a very specific agonist for mGluR2 with no reactivity with mGluR1.

We used this compound to distinguish whether the incorporation of the mGluR2 sequence in the chimeric receptors generates the ligand-selective property characteristic of mGluR2 or alternatively still retains the property of mGluR1. The result of this analysis is presented in Fig. 4. Although this experiment was carried out at a single concentration of DCG-IV (100 μM) because of its availability, it is clear from this analysis that the TQ3-TQ6 chimeric receptors acquire the reactivity with DCG-IV, whereas QT5, like the native mGluR1, shows no reactivity with this compound. This analysis thus explicitly demonstrated that about one-half of the amino-terminal extracellular domain is a critical determinant that confers an agonist selectivity of mGluR2. Interestingly, DCG-IV was not reactive with the TQ2 chimeric receptor, indicating that the amino-terminal sequence up to site N is not sufficient to exchange a different agonist selectivity between mGluR1 and mGluR2.

**DISCUSSION**

The mGluR family consisting of at least six different subtypes shows common structural architectures comprised of an unusually large extracellular domain preceding the seven transmembrane segments. mGluR1 and mGluR2 show similar affinities for L-glutamate but distinctive reactivities with quisqualate and tACPD. In this investigation, we attempted to assign structural determinants responsible for the different agonist selectivities between mGluR1 and mGluR2 by construction and expression of a series of chimeric receptors between these two receptor subtypes. Many chimeric receptors constructed responded to L-glutamate but lowered high-affinity responsiveness to L-glutamate by more than one order in magnitude. However, there was a distinct pattern in the reactivities with quisqualate and tACPD among the different series of chimeric receptors. Replacement of the amino-terminal portion up to site A of mGluR1 with the corresponding portion of mGluR2 in the TQ series of chimeric receptors (TQ3–TQ6) showed the reactivity with tACPD but not with quisqualate. In contrast, the QT5 receptor, in which the large amino-terminal portion was retained with the mGluR1 sequence, reacted with quisqualate more efficiently than L-glutamate but lost the ability to interact with tACPD. Thus, although the native mGluR1 showed the ability to respond to tACPD, the effective responsiveness to tACPD in the TQ3–TQ6 receptors was interpreted to indicate that incorporation of at least about one-half of the amino-terminal portion of mGluR2 generates the agonist selectivity characteristic of this receptor subtype. This interpretation was confirmed by the reactivity of these receptors and not the native mGluR1 or QT5 with DCG-IV, which is a specific agonist for mGluR2. Thus, the present investigation demonstrated that about one-half of the amino-terminal portion is critical in determining the agonist selectivity of mGluR. It is curious that many of the QT series of chimeric receptors are nonfunctional despite the fact that most of the reciprocal TQ series of chimeric receptors are actively expressed in *Xenopus* oocytes. Similar functional defects were observed for some of the chimeric formations in other G protein-coupled receptors (Kobilka et al., 1988; Nagayama et al., 1991; Yokota et al., 1992). This defect hampered the assignment of more limited domains important for agonist selectivity of mGluR. Because TQ2 and TQ3 differed only in the sequence between site N and site A but showed distinctive patterns in the responses to DCG-IV and other agonists, we constructed an additional chimeric receptor in which the sequence from site N to site A of mGluR1 was substituted by the corresponding region of mGluR2. However, the resultant chimeric receptor failed to respond to any of the agonists (data not shown). In our separate experiments, we raised

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2 Y. Hayashi and S. Nakamichi, unpublished observation.
antibodies against limited portions of the extracellular domain along the sequence of mGluR1. Among these antibodies, we found that an antibody raised against a restricted portion (amino acid residues 177-341 of mGluR1) covering the sequence from site C to site A is capable of inhibiting the glutamate-stimulated phosphatidylinositol hydrolysis of mGluR1 expressed in Chinese hamster ovary cells. This finding thus strongly supported our conclusion that the amino-terminal portion of the extracellular domain of mGluR1 is critical in evoking the characteristic response of this receptor.

The structural basis for ligand binding and selectivity of G protein-coupled receptors has been studied in different members of this receptor family. In the G protein-coupled receptors for small molecule ligands such as catecholamines and acetylcholine, several transmembrane domains are important for determining the agonist and antagonist selectivities by forming a ligand binding pocket (Frielle et al., 1988; Kobilka et al., 1988; Kubo et al., 1988). In the receptors for glycohormones such as lutropin-choriogonadotropin and thyrotropin, the large extracellular domains are responsible for binding these hormones and for conferring a high affinity (Xie et al., 1990; Nagayama et al., 1991), although the transmembrane segments are also partly involved in the recognition of these hormones (Ji and Ji, 1991a, 1991b). In the receptors for small peptides, both transmembrane segments and extracellular domains serve as determinants for the high-affinity interaction with these ligands (Yokota et al., 1992; Perez et al., 1993). Thus, mGluR seems to differ from any of the above modes of agonist selectivity determination of the G protein-coupled receptors.

The simple explanation for the involvement of the extracellular domain in the agonist selectivity is that the extracellular domain of mGluR1 represents a glutamate binding site that recognizes different agonists and thus confers agonist selectivity. In this case, the binding of an agonist to the extracellular domain could induce a conformational change that is transmitted across membrane-spanning domains and then activates a G protein at the cytoplasmic side. Alternatively, the membrane-spanning domains of mGluR1 may be more directly involved in signal transmission of ligand binding. In the case of the thrombin receptors, it has been reported that thrombin cleaves an amino-terminal extension of this receptor and creates a new amino terminus that is tethered to the binding pocket of the receptor and thus activates this receptor (Vu et al., 1991). Analogous to the thrombin receptor, glutamate binding to the extracellular domain may generate a new conformation that allows the amino-terminal portion to interact with a binding pocket situated within the membrane-spanning domains. In this investigation, however, it remained to be determined whether the amino-terminal extracellular domain indeed serves as a glutamate-binding site. Therefore, a different possibility is that a primary glutamate-binding site is situated within the transmembrane segments, but this interaction is strengthened by a cooperative action of the amino-terminal extracellular domain. To address the question of whether the transmembrane segments of mGluR1 are capable of binding L-glutamate, we made a new receptor that deleted a large amino-terminal portion (residues 62-590) of mGluR1 and examined the ability of this deleted receptor to respond to agonists in Xenopus oocytes. However, we failed to detect any responses, and all three possibilities described above remain to be determined.

In conclusion, mGluR1 is unique in its mechanism responsible for determining agonist selectivity, reflecting a peculiar amino-terminal structure of this receptor family. This uniqueness raises an interesting question regarding whether mGluR1 is a representative that uses common and combined ligand-binding mechanisms observed for other G protein-coupled receptors or is an evolutionary specialization in the family of the G protein-coupled receptors.

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