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European Journal of Pharmacology 300 (1996) 151–154

ejp

Short communication

# Functional coupling between metabotropic glutamate receptors and G proteins in rat brain membranes

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Received 6 November 1995; revised 4 January 1996; accepted 9 January 1996

**Abstract**

Functional activation of GTP-binding (G) proteins coupled with metabotropic glutamate receptors was evaluated in rat brain membranes. L-Glutamate stimulated the high-affinity GTPase activity in cerebral cortical, hippocampal, and striatal membranes with a mean concentration eliciting a half-maximal response ($EC_{50}$) of 4.8, 1.6, and 4.9 $\mu$M, respectively. The enzyme activity in cerebral cortical membranes was also stimulated by (2*S*,1'*S*,2'*S*)-2-(carboxycyclopropyl)glycine (L-CCG-I) with a mean $EC_{50}$ of 0.90 $\mu$M, but not by L-2-amino-4-phosphonobutyrate (L-AP4) up to 10 $\mu$M. This method opens up a strategy for investigation of functional coupling between Group II metabotropic glutamate receptors and G proteins in native brain membranes.

*Keywords:* Excitatory amino acid; Metabotropic glutamate receptor; GTP hydrolysis; G-protein; Adenylyl cyclase; Cerebral cortex

## 1. Introduction

Excitatory amino acids such as glutamate and aspartate mediate cellular effects via multiple receptor types in the central nervous system. These receptors can be classified into two major groups termed 'ionotropic' and 'metabotropic' receptors (Hollmann and Heinemann, 1994). The ionotropic glutamate receptors, i.e., *N*-methyl-D-aspartate (NMDA), $\alpha$-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA), and kainate receptors, are ligand-gated channels, whereas metabotropic glutamate receptors are coupled to intracellular effector proteins (phospholipase C, adenylyl cyclase, or 3',5'-cyclic GMP phosphodiesterase) via heterotrimeric guanine nucleotide-binding regulatory (G) proteins. Molecular cloning studies have revealed the existence of at least eight subtypes of metabotropic glutamate receptors so far (Pin and Duvoisin, 1995). Based on their amino acid sequence homology, pharmacological characteristics, and coupled intracellular second messenger generating systems, these metabotropic glutamate receptors have been classified into three groups (Group I, II, and III). Group I comprises the $mGlu_1$ and $mGlu_5$ receptors, Group II, the $mGlu_2$ and $mGlu_3$ receptors, and Group III, all the others (Pin and Duvoisin, 1995).

All metabotropic glutamate receptors have seven hydrophobic putatively transmembrane domains characteristic of G protein-coupled receptors. When transfected and expressed in the cultured cell lines, Group I receptors stimulate phospholipase C, resulting in phosphoinositide turnover and $Ca^{2+}$ release from internal stores. On the other hand, it has been shown that Group II and Group III receptors are negatively coupled to adenylyl cyclase. Although the involvement of G proteins in these responses has been demonstrated indirectly by using pertussis toxin (islet activating protein, IAP), there has been no report in which functional interaction between metabotropic glutamate receptors and G proteins in the central nervous system is described. In the present study, we have reported the activation of G protein function by L-glutamate through Group II metabotropic glutamate receptors in membranes prepared from rat brain.

## 2. Materials and methods

### 2.1. Membrane preparation

Male Sprague-Dawley rats (weighing 200–250 g) were decapitated and their brains were quickly removed. The cerebral cortical, hippocampal, and striatal tissue dissected from each rat were homogenized in 5 ml of ice-cold TED

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*PII* S0014-2999(96)00033-7

buffer (5 mM Tris-HCl, 1 mM EDTA, 1 mM dithiothreitol, pH 7.4) containing 10% (w/v) sucrose using a motor-driven Teflon/glass tissue grinder (20 strokes). The homogenate was centrifuged at $1000 \times g$ for 10 min, and the supernatant was kept on ice while the pellet was resuspended in 5 ml of TED/sucrose buffer and centrifuged again at $1000 \times g$ for 10 min. The combined supernatant was then centrifuged at $9000 \times g$ for 20 min, and the pellet was resuspended in 10 ml of TED buffer followed by another centrifugation at $9000 \times g$ for 20 min. The resulting pellet was resuspended in 10 ml of TED buffer and maintained on ice for 30 min. Finally, the suspension was centrifuged at $35000 \times g$ for 10 min, and the pellet was resuspended in 3.0 ml (cerebral cortex), 1.5 ml (hippocampus), or 1.0 ml (striatum) of 50 mM Tris-HCl buffer (pH 7.4). This homogenate (1.6–3.2 mg protein/ml) was quickly frozen on powdered dry ice and stored at −80°C until use.

### 2.2. High-affinity GTPase assay

GTP hydrolysis was determined by measuring $^{32}P_i$ released from [$\gamma$-$^{32}$P]GTP as described previously in detail (Odagaki and Fuxe, 1995a, b, c; Odagaki et al., 1995). In brief, the thawed membranes were diluted in 50 mM Tris-HCl and an aliquot (4–8 $\mu$g protein) was incubated at 30°C for 15 min in the assay mixture (final 100 $\mu$l) containing the following constituents: 50 mM Tris-HCl (pH 7.4), 0.3 $\mu$M [$\gamma$-$^{32}$P]GTP, 2 mM $MgCl_2$, 0.5 mM ATP, 0.5 mM adenylylimidodiphosphate, 5 mM phosphocreatine, creatine phosphokinase (50 U/ml), bovine serum albumin (50 $\mu$g), 0.1 mM EDTA, 0.2 mM EGTA, 0.2 mM dithiothreitol, 0.5 mM cyclic AMP, 0.5 mM 3-isobutyl-1-methylxanthine, and 100 mM NaCl. The enzyme reaction was stopped by chilling the tubes followed by the addition of 500 $\mu$l of 20 mM phosphoric acid containing 5% (w/v) activated charcoal. After a centrifugation of the tubes at $11000 \times g$ for 10 min, an aliquot (200 $\mu$l) from the supernatant fraction was mixed with 5 ml of scintillation cocktail, and the radioactivity was counted by liquid scintillation spectrometer. The high-affinity GTPase activity was calculated by subtracting the amount of $^{32}P_i$ released from [$\gamma$-$^{32}$P]GTP in the presence of 100 $\mu$M unlabeled GTP from the total activity.

### 2.3. Chemicals

[$\gamma$-$^{32}$P]GTP (30 Ci/mmol) was purchased from New England Nuclear (Boston, MA, USA). L-Glutamate was from Kanto Chemical Co. (Tokyo, Japan), (2*S*,1′*S*,2′*S*)-2-(carboxycyclopropyl)glycine (L-CCG-I) from Tocris Cookson (Bristol, UK), and AMPA hydrobromide from Research Biochemical Int. (Natick, MA, USA). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

## 3. Results

The concentration-response curves for L-glutamate-stimulated high-affinity GTPase activity in the cerebral cortical membranes are presented in Fig. 1A. L-Glutamate increased the high-affinity GTPase activity in a concentration-dependent and saturable manner. The concentration of L-glutamate eliciting the half-maximal response ($EC_{50}$) obtained from each experiment was logarithmically transformed and averaged to give the mean value of 4.8 $\mu$M (Table 1). The mean value (±S.E.M.) of the maximal percent stimulation above basal activity ($\%E_{max}$) was 32.3

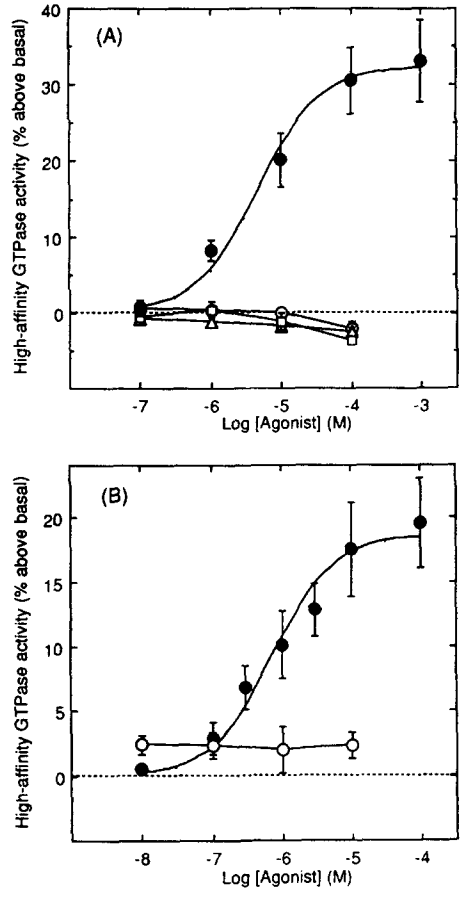


Fig. 1. Effect of glutamate receptor agonists on the high-affinity GTPase activity in cerebral cortical membranes. Hydrolysis of 0.3 $\mu$M [$\gamma$-$^{32}$P]GTP was measured after incubation of the membranes at 30°C for 15 min in the absence and presence of increasing concentrations of an agonist. (A) Stimulation of high-affinity GTPase activity by L-glutamate (●), NMDA (○), AMPA (□), and kainate (△). Results are given as percent increase above basal unstimulated high-affinity GTPase activity (1043 ± 120, 737 ± 44, 1064 ± 154, and 743 ± 50 pmol/mg protein/15 min for L-glutamate-, NMDA-, AMPA-, and kainate-stimulated activities, respectively). Values are means ± S.E.M. of 4 separate experiments, each performed in duplicate. (B) Stimulation of high-affinity GTPase activity by L-CCG-I (●) and L-AP4 (○). Results are given as percent increase above basal unstimulated high-affinity GTPase activity (923 ± 99 and 950 ± 98 pmol/mg protein/15 min for L-CCG-I- and L-AP4-stimulated activities, respectively). Values are means ± S.E.M. of 4–5 separate experiments, each performed in duplicate.

Table 1
Effect of L-glutamate on the high-affinity GTPase activity in rat brain membranes

| Brain region | $n$ | $-\text{Log } EC_{50}$ | Mean $EC_{50}$ ($\mu$M) | $\%E_{max}$ |
|---|---|---|---|---|
| Cerebral cortex | 4 | $5.32 \pm 0.06$ | 4.8 | $32.3 \pm 4.9$ |
| Hippocampus | 4 | $5.81 \pm 0.13$ | 1.6 | $17.2 \pm 2.2$ |
| Striatum | 4 | $5.31 \pm 0.13$ | 4.9 | $19.0 \pm 1.7$ |

Hydrolysis of 0.3 $\mu$M [$\gamma$-$^{32}$P]GTP was measured in the absence and presence of increasing concentrations of L-glutamate. Values are means ± S.E.M. of 4 separate experiments, each performed in duplicate. Basal unstimulated high-affinity GTPase activities were $1043 \pm 120$, $644 \pm 74$, and $713 \pm 55$ pmol/mg protein/15 min in cerebral cortex, hippocampus, and striatum, respectively.

($\pm 4.9$)%. The high-affinity GTPase activity was also stimulated by L-glutamate in hippocampal and striatal membranes with mean $EC_{50}$ values of 1.6 and 4.9 $\mu$M, respectively (Table 1). The mean $\%E_{max}$ values in both membranes ($17.2 \pm 2.2\%$ and $19.0 \pm 1.7\%$ in hippocampus and striatum, respectively) were less than that in the cerebral cortical membranes (Table 1). In contrast with the stimulatory effect of L-glutamate, none of the ionotropic glutamate receptor agonists (NMDA, AMPA, or kainate) stimulated at all the high-affinity GTPase activity in the cerebral cortical membranes within a broad concentration range of 0.1–100 $\mu$M (Fig. 1A).

L-CCG-I stimulated the enzyme activity in the cerebral cortical membranes with a mean $EC_{50}$ value of 0.90 $\mu$M ($-\log EC_{50} = 6.05 \pm 0.10$) and with a $\%E_{max}$ of $18.7 \pm 3.2\%$ (Fig. 1B). In contrast, L-2-amino-4-phosphonobutyrate (L-AP4) was totally ineffective up to 10 $\mu$M (Fig. 1B).

## 4. Discussion

In the present study, we have revealed for the first time that G proteins can be functionally activated by L-glutamate in the brain membranes by virtue of the assay for agonist-induced high-affinity GTPase activity. Heterotrimeric G proteins, composed of $\alpha$, $\beta$, and $\gamma$ subunits, are functionally regulated by guanine nucleotides such as GTP and GDP. Thus, binding of an agonist to the cell-surface receptor facilitates a GTP–GDP exchange reaction on the $\alpha$ subunit, and the activated form of $\alpha$ ($\alpha_{GTP}$) dissociates from $\beta\gamma$ subunits and interacts with effector molecules such as adenylyl cyclase. The GTP on the $\alpha$ subunit is then hydrolysed to GDP and $P_i$ by the intrinsic GTPase, and $\alpha_{GDP}$ reassociates with $\beta\gamma$ subunits to end the activation cycle (Gilman, 1987). Based on these fundamental characteristics, several methods have been devised to detect the receptor-mediated activation of G protein function. Agonist-induced high-affinity GTPase activity serves as a useful strategy for the investigation of receptor-mediated activation of G proteins, in particular the IAP-sensitive $G_i$ subfamily (Milligan, 1988). Indeed, we succeeded recently in detecting an increase in high-affinity GTPase activity by several neurotransmitters which activate receptors coupled to adenylyl cyclase inhibition via IAP-sensitive G proteins (Odagaki and Fuxe, 1995a, b, c; Odagaki et al., 1995). The fact that metabotropic glutamate receptors are coupled not only to phosphoinositide turnover but also to adenylyl cyclase inhibition in cultured neurons (Prezeau et al., 1992, 1994; Ambrosini et al., 1995), in dispersed hippocampal cells and hippocampal membranes as well (Itano et al., 1992), and in hippocampal slices (Schoepp et al., 1992) encouraged us to investigate metabotropic glutamate receptor-mediated high-affinity GTPase activity using brain membranes.

The involvement of ionotropic glutamate receptors in this response can be evidently excluded, since the high-affinity GTPase activity was not altered at all by any of ionotropic glutamate receptor agonists examined, i.e., NMDA, AMPA, or kainate, even at high concentrations ($\sim 100$ $\mu$M). It is, therefore, suggested that the L-glutamate-stimulated high-affinity GTPase activity presented in this report is most likely mediated by the metabotropic glutamate receptors.

As already mentioned, metabotropic glutamate receptors have been divided into at least eight structurally different subtypes by molecular cloning (Pin and Duvoisin, 1995). However, they can be classified pharmacologically into only three categories, namely, Group I, II, and III (Pin and Duvoisin, 1995). In the present study, L-AP4, which has been known as a potent agonist for Group III receptors but not for Group I or Group II receptors (Pin and Duvoisin, 1995), failed to stimulate the high-affinity GTPase activity in the cerebral cortical membranes up to a concentration of 10 $\mu$M. As effects of higher concentrations of L-AP4 were not examined, the possibility of involvement of the $mGlu_7$ receptor, a subtype of Group III metabotropic glutamate receptors (Okamoto et al., 1994), cannot be completely excluded. The $EC_{50}$ of L-AP4 was reported to be 160 $\mu$M as determined by inhibition of forskolin-stimulated cyclic AMP accumulation in Chinese hamster ovary cells expressing $mGlu_7$ receptor (Okamoto et al., 1994). However, this possibility seems unlikely, since it was shown in our preliminary experiment that the activity is not stimulated by broad concentrations (1–1000 $\mu$M) of L-serine-*O*-phosphate, which has been reported to be another agonist for the $mGlu_7$ receptor, with an $EC_{50}$ value of 160 $\mu$M (Okamoto et al., 1994). Then, it is most likely that L-glutamate-stimulated high-affinity GTPase activity in the cerebral cortical membranes is mediated by metabotropic glutamate receptor subtype(s) other than Group III.

Additionally, the high-affinity GTPase activity in the cerebral cortical membranes was stimulated by L-CCG-I in a concentration-dependent manner with a submicromolar $EC_{50}$ value. Using the Chinese hamster ovary cells in which the $mGlu_1$ (Group I), $mGlu_2$ (Group II), and $mGlu_4$

(Group III) receptor subtypes were expressed, it has been shown that L-CCG-I is a potent agonist for the $mGlu_2$ receptor with an $EC_{50}$ value of 0.3 $\mu$M as determined by the receptor-mediated inhibition of forskolin-stimulated cyclic AMP formation and that it has an $EC_{50}$ value of 50 $\mu$M for both the $mGlu_1$ receptor-mediated phosphatidylinositol hydrolysis and the $mGlu_4$ receptor-mediated inhibition of forskolin-stimulated cyclic AMP formation (Hayashi et al., 1992). Taken together, the pharmacological profile of the metabotropic glutamate receptors-mediated high-affinity GTPase activity in the cerebral cortical membranes presented in this report indicates that the Group II subtypes ($mGlu_2$ and/or $mGlu_3$ receptors) are most likely involved in the response. This notion is quite consistent with the feature of our assay system for the agonist-induced high-affinity GTPase activity. Thus, all previous results have shown that this method is favorable for functional coupling between the $G_i$ subfamily and inhibitory receptors such as dopamine $D_2$ (Odagaki and Fuxe, 1995b), pirenzepine-insensitive muscarinic (Odagaki and Fuxe, 1995b), $GABA_B$ (Odagaki et al., 1995), 5-$HT_{1A}$ (Odagaki and Fuxe, 1995c), and adenosine $A_1$ (Odagaki and Fuxe, 1995a) receptors, all of which have been known to mediate adenylyl cyclase inhibition. It has been known that Group I metabotropic glutamate receptors are coupled to phosphoinositide/$Ca^{2+}$ metabolism whereas Group II and III metabotropic glutamate receptors are negatively coupled to adenylyl cyclase activity (Pin and Duvoisin, 1995).

In conclusion, we present here for the first time the functional activation of G proteins coupled with metabotropic glutamate receptors in brain membranes. This response is mediated probably through the Group II metabotropic glutamate receptors which are coupled with adenylyl cyclase inhibition at least in the rat cerebral cortex. Although the question of the exact receptor subtype(s) and G protein subclass(es) responsible for L-glutamate-stimulated high-affinity GTPase activity in the cerebral cortex and other brain regions remains to be answered in future more detailed studies, this method opens up a strategy for investigating directly the functional coupling between metabotropic glutamate receptors and G proteins in native brain membranes.

## Acknowledgements

The preparation costs of this article were supported by the memorial fund of a deceased psychiatrist, Dr. Kozo Itoh.

## References

Ambrosini, A., L. Bresciani, S. Fracchia, N. Brunello and G. Racagni, 1995, Metabotropic glutamate receptors negatively coupled to adenylate cyclase inhibit *N*-methyl-D-aspartate receptor activity and prevent neurotoxicity in mesencephalic neurons in vitro, Mol. Pharmacol. 47, 1057.

Gilman, A.G., 1987, G proteins: transducers of receptor-generated signals, Annu. Rev. Biochem. 56, 615.

Hayashi, Y., Y. Tanabe, I. Aramori, M. Masu, K. Shimamoto, Y. Ohfune and S. Nakanishi, 1992, Agonist analysis of 2-(carboxycyclopropyl)glycine isomers for cloned metabotropic glutamate receptor subtypes expressed in Chinese hamster ovary cells, Br. J. Pharmacol. 107, 539.

Hollmann, M. and S. Heinemann, 1994, Cloned glutamate receptors, Annu. Rev. Neurosci. 17, 31.

Itano, Y., T. Murayama, Y. Kitamura and Y. Nomura, 1992, Glutamate inhibits adenylate cyclase activity in dispersed rat hippocampal cells directly via an *N*-methy-D-aspartate-like metabotropic receptor, J. Neurochem. 59, 822.

Milligan, G., 1988, Techniques used in the identification and analysis of function of pertussis toxin-sensitive guanine nucleotide binding proteins, Biochem. J. 255, 1.

Odagaki, Y. and K. Fuxe, 1995a, Functional coupling between $A_1$ adenosine receptors and G-proteins in rat hippocampal membranes assessed by high-affinity GTPase activity, Br. J. Pharmacol. 116, 2691.

Odagaki, Y. and K. Fuxe, 1995b, Functional coupling of dopamine $D_2$ and muscarinic cholinergic receptors to their respective G proteins assessed by agonist-induced activation of high-affinity GTPase activity in rat striatal membranes, Biochem. Pharmacol. 50, 325.

Odagaki, Y. and K. Fuxe, 1995c, Pharmacological characterization of the 5-hydroxytryptamine-1A receptor-mediated activation of high-affinity GTP hydrolysis in rat hippocampal membranes, J. Pharmacol. Exp. Ther. 274, 337.

Odagaki, Y., S. Dasgupta and K. Fuxe, 1995, Additivity and non-additivity between dopamine-, norepinephrine-, carbachol- and GABA-stimulated GTPase activity, Eur. J. Pharmacol. Mol. Pharmacol. 291, 245.

Okamoto, N., S. Hori, C. Akazawa, Y. Hayashi, R. Shigemoto, N. Mizuno and S. Nakanish, 1994, Molecular characterization of a new metabotropic glutamate receptor $mGluR_7$ coupled to inhibitory cyclic AMP signal transduction, J. Biol. Chem. 269, 1231.

Pin, J.-P. and R. Duvoisin, 1995, The metabotropic glutamate receptors: structure and functions, Neuropharmacology 34, 1.

Prezeau, L., O. Manzoni, V. Homburger, F. Sladeczek, K. Curry and J. Bockaert, 1992, Characterization of a metabotropic glutamate receptor: direct negative coupling to adenylyl cyclase and involvement of a pertussis toxin-sensitive G protein, Proc. Natl. Acad. Sci. USA 89, 8040.

Prezeau, L., J. Carrette, B. Helpap, K. Curry, J.P. Pin and J. Bockaert, 1994, Pharmacological characterization of metabotropic glutamate receptors in several types of brain cells in primary cultures, Mol. Pharmacol. 45, 570.

Schoepp, D.D., B.G. Johnson and J.A. Monn, 1992, Inhibition of cyclic AMP formation by a selective metabotropic glutamate receptor agonist, J. Neurochem. 58, 1184.