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Characterization of metabotropic glutamate receptors coupled to a pertussis toxin sensitive G-protein in bovine brain coated vesicles

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Glutamate metabotropic receptors (mGluRs) in bovine brain coated vesicles have been characterized by pharmacological and kinetic binding experiments. Saturation experiments revealed a single binding site with a K_d = 607.9 ± 78.5 nM and a B_{max} = 6.45 ± 0.88 pmol/mg protein. The specific binding of L-[^{3}H]glutamate to mGluRs is regulated by guanine nucleotides. Guanosine-5'-triphosphate (GTP; 100 μM) shifts the agonist competition curves to the right, increasing the IC_{50} values. Pertussis toxin treatment produces a pharmacological binding profile for quisqualate similar to that obtained in the presence of 100 μM GTP. These results indicate the presence of metabotropic glutamate receptors in coated vesicles and its coupling to a pertussis toxin sensitive G-protein.

Coated vesicle; Metabotropic glutamate receptor; G-protein; *trans*-(±)-1-Aminocyclopentane 1,3-dicarboxilic acid; Quisqualic acid; Radioligand binding

1. INTRODUCTION

Glutamate (Glu), a major excitatory neurotransmitter, plays an important role in neuronal plasticity and neurotoxicity and is thought to be involved in many neuronal functions including memory acquisition, learning and some degenerative disorders such epilepsy and stroke [1,2]. The functional diversity of glutamate results from the presence of a wide variety of receptors, which can be categorized into two distinct groups, termed ionotropic and metabotropic (mGluR) [1]. Both kinds of receptors are functionally and pharmacologically different. The ionotropioc glutamate receptors belong to the superfamily of receptor channels, and have been termed according to their most selective agonists (NMDA, AMPA and kainic acid) [3,4]. The metabotropic glutamate receptors are coupled to G-proteins and evoke a wide variety of intracellular signal transduction pathways [5,6]. Recently, cDNA clones for five different subtypes of the mGluR have been described [7–9], suggesting the existence of a family of mGluRs which are expressed heterogeneously in the brain [8,10,11]. It is known that mGluR1 and mGluR5 are linked to the PI hydrolysis/Ca^{2+} cascade [7,9,12] and mGluR2 is coupled to an inhibitory cAMP cascade [8,13,14]; nevertheless the precise signalling pathways of these receptors remain to be elucidated. On the other hand, the pharmacological characterization of mGluRs is proving very difficult because of the lack of specific radioactive ligands. It is known that these receptors can be activated by quisqualic acid (QA), ibotenate (IBO) and more selectively by *trans*-(±)-1-aminocyclopentane 1,3-dicarboxylic acid (t-ACPD) [15,16]. All these reports provide evidence of a novel family of seven transmembrane G-protein-coupled receptors.

Macromolecules are taken up into eukaryotic cells by endocytosis. One of the mechanisms for this process is mediated by cell surface receptors that cluster into clathrin-coated domains in the plasma membrane [17,18]. Receptors are collected in these regions either constitutively or after binding ligand and internalized by invagination and pinching off of clathrin-coated vesicles [19]. Some reports have shown the presence in coated vesicles of receptors that are coupled to guanylnucleotide-binding proteins, like opioid receptors [20], β-adrenergic receptors [21], muscarinic cholinergic receptors [22] and A_1-adenosine receptors [23] indicating that these receptors might be internalized via a similar mechanism [24]. The precise pathway of receptor sequestration or internalization is unclear so far, but the presence of several G-protein coupled receptors in coated vesicles suggests that these organelles may be involved in receptor translocation and redistribution. In a previous report [25] we have described the presence of glutamate receptors (ionotropic and metabotropic) in bovine brain coated vesicles. In this article we have characterized them by kinetic and pharmacological binding experiments and the effect of GTP on the mGluR specific binding and its putative coupling to a pertussis toxin-sensitive G-protein has been studied.

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2. MATERIALS AND METHODS

2.1. *Materials*

L-[^{3}H]Glutamic acid (54.7 Ci/mmol) was obtained from Dupont-NEN (Boston, MA, USA). Pertussis toxin was from List Biological Laboratories. Guanosine triphosphate was purchased from Boehringer Mannheim. L-Glutamic acid, IBO, QA and other reactives were from Sigma (St. Louis, MO, USA). t-ACPD was from Tocris Neuramin (Essex, UK). The Sephacryl S-1000 gel filtration column and Ficoll 400 were from Pharmacia (Sweden). All other reagents were of analytical grade.

2.2. *Preparation of bovine brain membranes and coated vesicles*

All steps were carried out at 4°C. Bovine brains were obtained at a local abattoir. For membrane preparation, 2 g of cerebral cortex were minced and homogenized in a buffer containing a mixture of protease inhibitors (100 μM PMFS, 7.3 mU/ml bacitracin), and a P_2 fraction was prepared as described by Recasens et al. [26], using 10 mM phosphate buffer, pH 7.5 [25]. Coated vesicles were purified from bovine cerebral cortex by Sephacryl S-1000 gel filtration according to a modification of several published procedures [23,27,28].

2.3. *Radioligand binding assays*

Metabotropic glutamate receptors were determined using L-[^{3}H]glutamate as radioligand. Binding assays were performed as previously reported [25,29,30]. Unless otherwise indicated, 70–100 μg of Triton-treated coated vesicles or other membrane fractions were incubated for 60 min at 22°C in the presence of 40 nM L-[^{3}H]glutamate, 100 μM AMPA, 100 μM NMDA and 100 μM KA in 10 mM potassium phosphate buffer, pH 7.4, to define total binding. Non-specific binding (cpm obtained in the presence of 100 μM of the specific ligand: QA, IBO, t-ACPD) was substracted from total binding to obtain specific binding. For competition experiments, different concentrations of the indicated specific agonists were used under the above conditions. Saturation analysis was performed using different concentrations of L-[^{3}H]glutamate (10 nM to 1,500 nM) in the presence of 100 μM AMPA, 100 μM NMDA and 100 μM KA (total binding), and in the presence of unlabeled glutamate at a concentration of 10^5 of the radioligand to obtain nonspecific binding K_d and B_{max} were determined by Scatchard analysis. All these experiments were performed in the absence and in the presence of 100 μM GTP.

2.4. *Pertussis toxin ADP-ribosylation*

Pertussis toxin ADP-ribosylation was carried out as described earlier [31]. Coated vesicles were incubated with preactivated pertussis toxin (1 μg PT/100 μg protein) or vehicle for 30 min at 30°C in a final volume of 1 ml of 100 mM potassium phosphate buffer, pH 7.4. After incubation, samples were centrifuged at 100,000 × g for 30 min and treated with Triton X-100 and used for binding experiments. In all these experiments, coated vesicles were rechromatographed through the Sephacryl S-1000 column to assure the purity of the preparation.

2.5. *Protein determination*

Protein concentration was determined by the method of Lowry et al. [32] using bovine serum albumin as standard.

2.6. *Sodium dodecyl sulfate polyacrylamide-gel electrophoresis*

Samples containing 50 μg protein were analyzed on polyacrylamide gradients gels (7.5–15%) containing 0.1 sodium dodecyl sulfate (SDS) [33].

3. RESULTS AND DISCUSSION

Bovine brain coated vesicles were obtained as described previously [23] after one or two passages on a Sephacryl S-1000 column to ensure purity. This method yields a coated vesicle preparation with minimal contamination with other subcellular fractions. Purity was assessed by several enzyme markers as described earlier [22,23]. Analysis by SDS-PAGE (Fig. 1) confirmed that peak II (lanes 3 and 4) contain mostly coated vesicles showing the clathrin heavy chain (M_r = 180,000) as the major component [34] as well as the light chains (M_r = 30,000–36,000) and the other two major family proteins (100,000–115,000; 50,000–55,000) implicated in clathrin assembly to the plasma membranes, termed adaptor proteins [35]. Lanes 1 and 2 correspond to the proteins of sample before S-1000 column and peak I, respectively. Peak I showed a small quantity of 180,000 clathrin heavy chain. Crude synaptic membranes (lane 5) as well as the former samples present a different electrophoretic pattern profile than coated vesicles. This agrees with our previous reports [23,25] and with other authors [17,28] who also found the same electrophoretic profiles, and besides they conclude that most of the bovine brain coated vesicles derive from neuronal cells and are implicated in the vesicular neuronal membrane traffic pathways [28]. To assess the presence of metabotropic glutamate receptors in bovine brain coated vesicles, L-[^{3}H]glutamate was used as radioli-

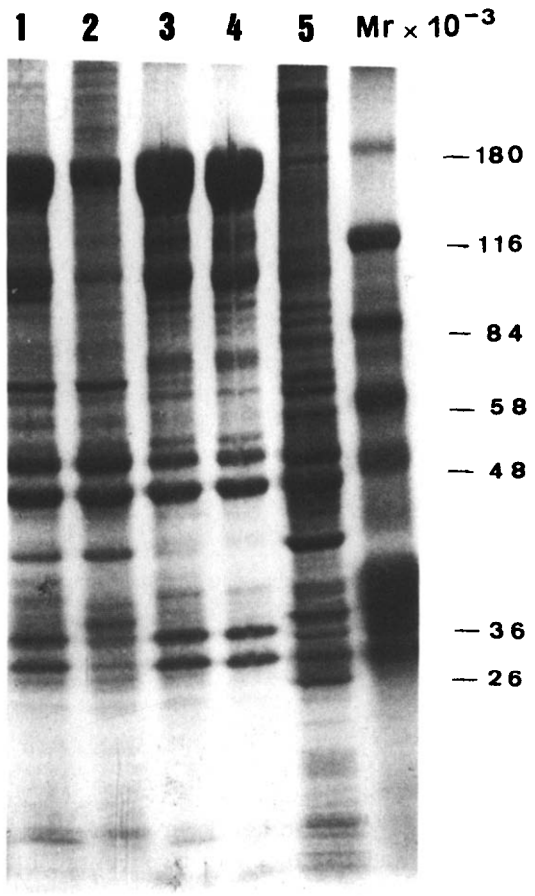

Fig. 1. SDS-PAGE of a coated vesicle preparation. Samples (50 μg of protein) of different fractions were loaded in a SDS-PAGE. Lane 1, sample before S-1000 column; lane 2, peak I; lane 3, coated vesicles (peak II); lane 4, rechromatographed coated vesicles; lane 5, bovine brain membranes.

Table I

Specific binding of L-[^{3}H]glutamate to metabotropic glutamate receptors in various subcellular fractions

Fractions	Specific binding (pmol/mg protein)
Bovine brain membranes	0.121 ± 0.031 (7)
Before S-1,000 column	0.215 ± 0.016 (3)
Peak I	0.443 ± 0.032 (4)
Peak II	0.666 ± 0.050 (6)

Different fractions were treated with 0.04% Triton X-100 and incubated in the presence of 40 nM [^{3}H]glutamate, 100 μM NMDA, 100 μM AMPA and 100 μM KA (total binding). Non-specific binding was determined in the presence of 100 μM t-ACPD. Data are means (±S.E.M.) of the experiments indicated in parentheses, performed in triplicate each using different preparations.

gand. Metabotropic glutamate receptors were measured in bovine brain membranes and different fractions obtained during coated vesicles purification. As it can be seen in Table I specific binding in peak II (coated vesicles) to mGluR using t-ACPD as specific agonist represents 550% of the binding found in the bovine brain membranes and 150% of the binding found in peak I. Taken into the account that the cross-contamination between peaks I and II was minimal [22,23] and that the electrophoretic pattern (Fig. 1) of bovine brain membranes and coated vesicles were clearly different, these results support the evidence of the presence of metabotropic glutamate receptors in bovine brain coated vesicles. Furthermore these data confirm our previous results where, under these conditions, the mGluRs found in bovine brain coated vesicles represented 45–50% of the total L-[^{3}H]glutamate binding sites [25].

To further characterize the mGluRs present in bovine brain coated vesicles and considering that these receptors are coupled to G-proteins [5,6], the kinetic and pharmacological characteristics in the absence and in the presence of GTP were studied. Fig. 2 shows the saturation binding experiments in the absence of guanine nucleotide. Scatchard plot (inset) indicates the presence of one binding site with a K_d value of 607.9 ± 78.5 nM and a B_{max} of 6.45 ± 0.88 pmol/mg protein. The K_d value is similar to the K_d reported earlier [25] for L-[^{3}H]glutamate binding sites and B_{max} represents 55% of the total glutamate receptors (ionotropic and metabotropic) found in coated vesicles [25]. When these studies were performed in the presence of 100 μM GTP (Table II) kinetics parameters (K_d and B_{max}) were increased. It is well known that receptors whose action is mediated through a G-protein show a lower affinity for its agonists in the presence of GTP. GTP produces a transition of the receptors from the high- to the low-

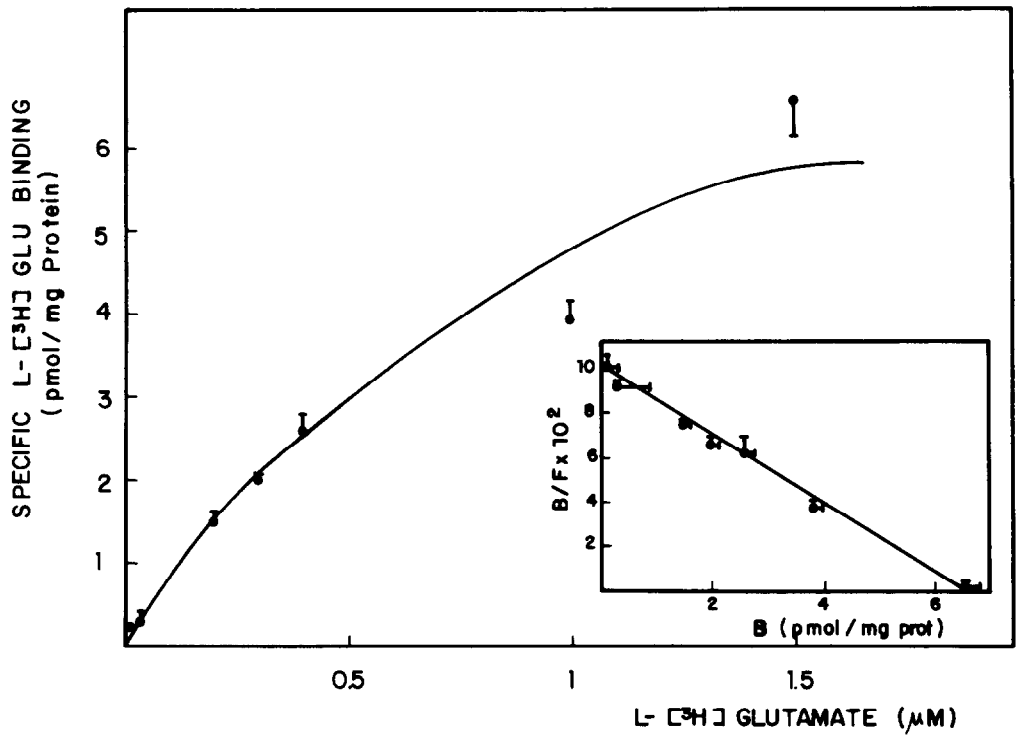

Fig. 2. Saturation binding curve for glutamate metabotropic receptors in bovine brain coated vesicles. Binding assays were performed as described in section 2. The data points are means (±S.E.M.) of four individual experiments made in triplicate, each using different coated vesicles preparation. Inset shows Scatchard plot analysis from the data of saturation curve giving a K_d = 607.9 ± 78.5 nM and a B_{max} = 6.45 ± 0.88 pmol/mg prot. At either radioligand concentration ≃50–55% of total L-[^{3}H]glutamate binding was specific. Total binding was between 0.6% and 1.2% of the total amount of radioactivity in the assay (maximum 300,000 cpm).

Table II

Saturation data for the L-[^{3}H]glutamate binding to mGluRs

	-GTP	+GTP
K_d (nM)	607.9 ± 78.5	1,440 ± 30
B_{max} (pmol/mg prot.)	6.45 ± 0.88	9.35 ± 1.5

Binding assays were performed as described in section 2. Data are obtained as described in the legend to Fig. 2.

affinity state [36]. In our conditions, it is possible that in the absence of GTP we only detect the binding sites corresponding to the low-affinity state; however, an increase in the B_{max} and K_d values are observed in the presence of GTP suggesting that a transition from the high- or middle- to the low-affinity state could take place in the presence of guanine nucleotides [37]. In agreement with these data, it has been reported that receptors in several agonists affinity states may be present depending on lipid environment [38]. The lack of specific radioligands make a better kinetic characterization of the mGluRs difficult.

When bovine brain coated vesicles were incubated in the absence or in the presence of 100 μM GTP and non-specific binding to mGluRs was obtained in the presence of 100 μM of the indicated agonists (Table III), specific binding was reduced by more than 50% in all cases except for L-glutamate. These results were confirmed with the competition binding experiments using different concentrations of specific agonists (t-ACPD, QA, IBO) (Fig. 3). The pharmacological profiles in the absence of GTP show that t-ACPD is the most specific agonist. These results agree with the pharmacological response of mGluR2 on the inhibition of cAMP formation [8], as well as with other reports which show that

Table III

Effect of GTP on the specific binding of metabotropic glutamate receptors in bovine brain coated vesicles

	Specific binding (pmol/mg protein)	
	-GTP	+GTP
Glutamate	0.726 ± 0.043 (100%)	0.575 ± 0.012 (79%)
Quisqualate	0.630 ± 0.058 (100%)	0.274 ± 0.077 (43%)
Ibotenate	0.585 ± 0.066 (100%)	0.258 ± 0.02 (44%)
t-ACPD	0.713 ± 0.022 (100%)	0.307 ± 0.03 (43%)

Triton-treated coated vesicles were incubated for 60 min at 22°C in the presence of 40 nM L-[^{3}H]glutamate, 100 μM NMDA, 100 μM AMPA and 100 μM KA in phosphate potassium buffer (total binding), in the absence or in the presence of 100 μM GTP. Non-specific binding was determined in the presence of 100 μM of the indicated agonists. Data are means (±S.E.M.) of four separated experiments performed in triplicate.

t-ACPD is a more potent agonist than QA and IBO in stimulating PI metabolism in different brain regions [6,15]. Moreover, when these experiments were performed in the presence of 100 μM GTP, the dose–response curves shifted to the right, increasing the IC_{50} values (Fig. 3). All these results confirm the coupling of the mGluRs found in bovine brain coated vesicles, to a G-protein.

As has been pointed out above, the existence of several forms of metabotropic glutamate receptors by pharmacological [13,16], electrophysiological [5,39] and cloning approaches [7–9] has been shown. Furthermore, it is well known that some of the effects of these receptors are pertussis toxin-sensitive [9,12,14,40] and some others are not [9,41,42], depending on the cell type studied. The effect of PTX on mGluR binding was investigated by treatment of bovine brain coated vesicles with

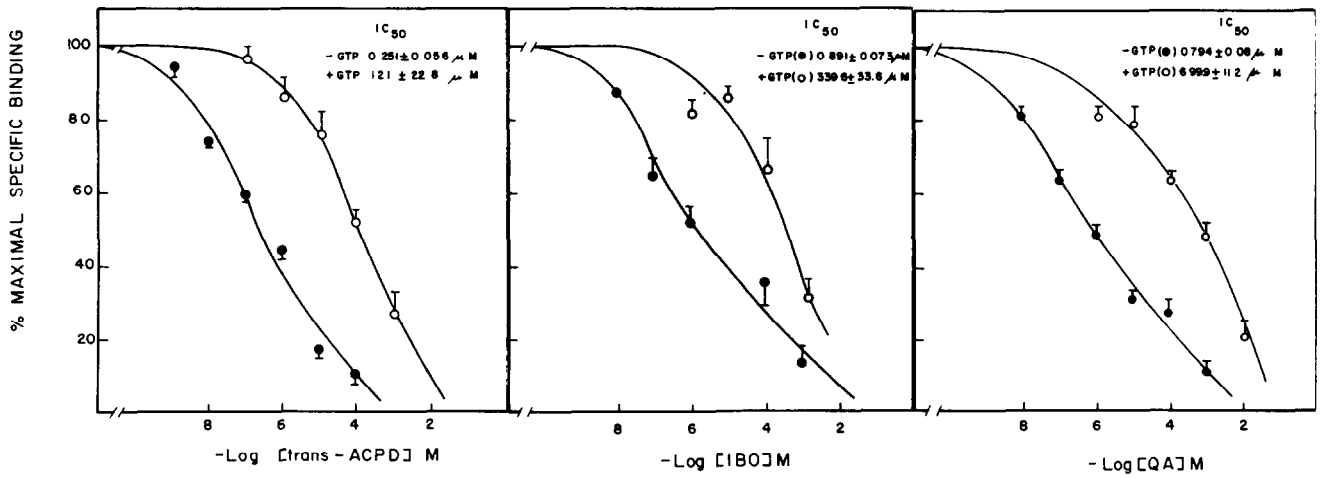

Fig. 3. Effect of GTP on agonists competition curves in bovine brain coated vesicles. Specific binding of L-[^{3}H]glutamate to mGluRs in the absence (●) or in the presence (○) of 100 μM GTP was determined at various concentrations of the indicated specific agonists. Results are expressed as percentage of maximal specific binding in each case. Binding was performed as described in section 2. The data points are obtained as described in the legend of Fig. 2. IC_{50} values are means (±S.E.M.) of four individual experiments made in triplicate. Maximal specific binding in each case is expressed in Table III.

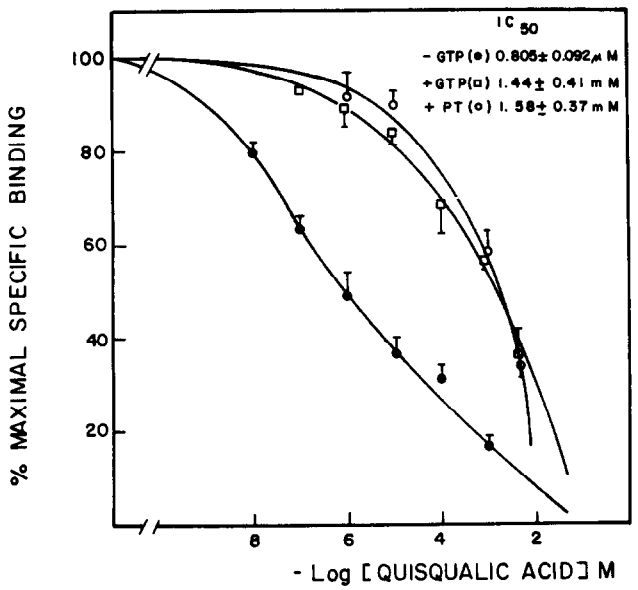

Fig. 4. Pertussis toxin effect on quisqualic acid competition curves in bovine brain coated vesicles. Specific binding of L-[^{3}H]glutamate to mGluRs in untreated (●,□) and treated (○) pertussis-toxin coated vesicles was determined in the absence (●,○) and in the presence of 100 μM GTP (□) at indicated concentrations of QA. Results are expressed as percentage of maximal specific binding in each case. Data points are obtained as described in the legend of Fig. 2. IC_{50} values are means (±S.E.M.) of four individual experiments made in triplicate. Maximal specific binding was (●) 0.712 ± 0.039 pmol/mg protein; (□) 0.363 ± 0.047 pmol/mg protein; (○) 0.346 ± 0.012 pmol/mg protein.

1 μg PTX/100 μg protein, prior to the QA competition binding experiments. Fig. 4 shows that PTX treatment produces the same effect as guanine nucleotides. The PTX competition curve shifts to the right, causing an appreciable increase of the IC_{50} value. Supporting these data, we have previously reported the presence of pertussis toxin substrates in bovine brain coated vesicles [43].

These results show that metabotropic glutamate receptors present in bovine brain coated vesicles are coupled to a pertussis toxin-sensitive G-protein. This, and the fact that a wide number of G-protein coupled receptors are present in coated vesicles [20–23] as well as other proteins implicated in signal transduction systems, such as adenylate cyclase [21,23], G-proteins [43] and phospholipase C [31], which suggests that these organelles may play a role in the translocation and/or the regulation of G-protein-coupled receptors [24,44].

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