Metabotropic glutamate receptor analogues inhibit p[NH]ppG-stimulated phospholipase C activity in bovine brain coated vesicles: involvement of a pertussis toxin-sensitive G-protein

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Guanosine 5'-[β -imido]triphosphate (p[NH]ppG)-stimulated phospholipase C (PLC) activity in bovine brain coated vesicles is inhibited by glutamate agonists. In the present study we show that quisqualic acid (QA), ($\pm$)-*trans*-1-aminocyclopentane-1,3-dicarboxylate (*trans*-ACPD), glutamic acid and ibotenic acid inhibited p[NH]ppG-stimulated PLC by 44, 41, 36 and 25% respectively. Carbachol also produced an inhibition of p[NH]ppG-stimulated PLC by 45%. The inhibition caused by *trans*-ACPD and QA was dose-dependent. DL-2-Amino-3-phosphonopropionic acid and (*RS*)- α -methyl-4-carboxyphenylglycine, specific antagonists of metabotropic glutamate receptors (mGluRs), abolished these inhibitory effects. *trans*-ACPD in-

hibition of p[NH]ppG-stimulated PLC was also observed in the presence of ionotropic glutamate receptor antagonists. When carbachol and QA or *trans*-ACPD were combined, additive inhibitory effects were observed. Preincubation of bovine brain coated vesicles with pertussis toxin abolished the inhibitory effects of mGluR analogues and carbachol on p[NH]ppG-stimulated PLC activity. The presence of G_{α} and pertussis toxin substrates, G_{α} and G_{α} subunits as well as PLC β_1 in bovine brain coated vesicles has been confirmed by immunoblot. These results support the coupling of mGluRs to a PLC in an inhibitory manner through a pertussis toxin-sensitive G-protein in bovine brain coated vesicles.

INTRODUCTION

Glutamate acts as a neurotransmitter in the central nervous system by interacting with specific membrane receptors. The functional diversity of glutamate results from the presence of multiple glutamate receptors which can be categorized into two main groups termed ionotropic and metabotropic receptors [1]. At least seven metabotropic glutamate receptors (mGluRs) have been described by molecular cloning [2–6]. These receptors are coupled to G-proteins and evoke a variety of functions through different signal transduction systems and effectors, including the phosphatidylinositol/ Ca^{2+} cascade and inhibition of adenylyl cyclase.

Receptor-mediated activation of phospholipase C (PLC) involves the participation of G-proteins. By analogy with the adenylyl cyclase system, it has been suggested that PLC may be regulated via distinct G-proteins, which could activate or inhibit through stimulatory or inhibitory receptors respectively [7]. In fact, a dual regulation of PLC activity by guanine nucleotides has been shown in rat cerebral cortical membranes [8] and bovine brain coated vesicles [9]. Members of the G_q family have been reported to mediate pertussis toxin-insensitive stimulation of PLC [10]. Inhibition of stimulated PLC activity by glutamate analogues [11,12] as well as by other agonists, such as adenosine [13], carbachol [14] or opioid agonists [15], have been reported. Depending on the system studied, these inhibitory effects on stimulated-PLC activity have been explained through an indirect mechanism, probably mediated by calcium [16] or by direct coupling of their receptors with the PLC effector system [14,15]. Recently, Litosch et al. [17] have demonstrated G-protein-mediated inhibition of PLC β_1 in bovine brain solubilized membrane

preparation. However, identification and/or direct correlation between receptor-mediated inhibition of PLC and any characterized G-protein remains to be elucidated.

The presence of mGluRs [18,19] and PLC activity [9] in bovine brain coated vesicles has been described, suggesting that these organelles may play a role in the translocation and/or regulation of the G-protein-coupled receptors, as other authors have pointed out [20,21]. In this report we present evidence for the coupling of mGluRs, found in bovine brain coated vesicles, to a PLC activity in an inhibitory manner through a pertussis toxin-sensitive G-protein.

MATERIALS AND METHODS

Materials

Phosphatidyl [2- 3H]inositol 4,5-bisphosphate ([3H]PtdInsP2) was purchased from Amersham or American Radiolabeled Chemicals Inc. (St Louis, MO, U.S.A.). [^{32}P]NAD $^{+}$ was from New England Nuclear. Pertussis toxin was from List Biological Laboratories. Non-hydrolysable guanine nucleotides were purchased from Boehringer-Mannheim. L-Glutamic acid, ibotenic acid (IB), quisqualic acid (QA) and other reagents were from Sigma. ($\pm$)-*trans*-1-Aminocyclopentane-1,3-dicarboxylate (*trans*-ACPD), DL-2-amino-3-phosphonopropionic acid (DL-AP3), (*RS*)- α -methyl-4-carboxyphenylglycine (MCPG) and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) were from Tocris Neuramin (Essex, U.K.). Sephacryl S-1000 gel-filtration column and Ficoll-400 were from Pharmacia (Sweden). (5*R*,10*S*)-(+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a,d*]cyclohepten-5-10-imine hydrogen maleate (MK-801) was from RBI (SEMAT, U.K.). Antibodies

Abbreviations used: CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; DL-AP3, DL-2-amino-3-phosphonopropionic acid; EAA, excitatory amino acids; p[NH]ppG, guanosine 5'-[β -imido]triphosphate; IB, ibotenic acid; mGluR, metabotropic glutamate receptor; MCPG, (*RS*)- α -methyl-4-carboxyphenylglycine; MK-801, (5*R*,10*S*)-(+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a,d*]cyclohepten-5-10-imine hydrogen maleate; PMSF, phenylmethanesulphonyl fluoride; PLC, phospholipase C; [3H]PtdInsP2, phosphatidyl [2- 3H]inositol 4,5-bisphosphate; QA, quisqualic acid; *trans*-ACPD, *trans*-1-aminocyclopentane-1,3-dicarboxylate.

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(AS/7, RM/1 and GC/2) were from Biotechnology Systems New England Nuclear Research Products (Boston, MA, U.S.A.). Anti-bovine PLC β_1 antibody was from Upstate Biotechnology Inc. (New York, NY, U.S.A.). All other reagents were of analytical grade.

Preparation of bovine brain membranes and coated vesicles

Bovine brain membranes were obtained as described by Recasens et al., [22] using 10 mM potassium phosphate buffer, pH 7.5. Cerebral cortex (2 g) were minced and homogenized (Dounce tissue grinder) in a buffer containing protease inhibitors [100 μ M phenylmethanesulphonyl fluoride (PMSF), 7.3 mIU/ml bacitracin]. P_2 fraction was obtained after centrifugation at 17000 g for 20 min. Membranes were lysed for 30 min at 4 °C and washed twice. Bovine brain coated vesicles were obtained by Sephacryl S-1000 gel filtration as described by Bar-Zvi and Branton [23] and modified by González-Calero et al. [24]. Briefly, 300 g of cortex brain tissue was homogenized in a Waring blender in a ratio of 1:2 (w/v) tissue:isolation buffer (10 mM MES/NaOH, 100 mM KCl, 1 mM EGTA, 0.5 mM $MgCl_2$, 0.1 mM dithiothreitol, 0.02% NaN_3 , 7.3 mIU/ml bacitracin and 0.1 mM PMSF, pH 6.5, at room temperature). Crude coated vesicles were obtained by centrifugation (40 min at 42000 g) in a Ficoll-sucrose solution (12.5% w/v Ficoll 400/12.5% sucrose) in isolation buffer, followed by centrifugation for 1 h at 100000 g . Crude coated vesicles (10 mg in 3 ml) were loaded onto a Sephacryl S1000 column (1.6 $\times$ 70 cm). Purified coated vesicles were finally obtained by centrifugation of Peak II as described previously [24]. This preparation yielded 6–8 mg of coated vesicles.

Preparation of stripped vesicles

Stripped vesicles were prepared as described by Georgieva-Hanson et al. [25]. Coated vesicles (2–5 mg) from Sephacryl S1000 column purification were incubated in 0.5 M Tris/HCl buffer, pH 7.8 (25 ml), for 3 h at 4 °C under gentle shaking conditions. The stripped vesicles were then sedimented for 1 h at 100000 g and resuspended in a suitable buffer.

PLC assay

PLC in brain membranes and coated vesicles was assayed using [3H]PtdInsP2 as exogenous substrate by the method described by Tiger et al. [26]. The packaging solvent was evaporated under an N_2 stream and the lipid was dissolved in 0.01% (0.25 mM) sodium deoxycholate in 50 mM Tris/HCl (pH 6.8) at a concentration of 0.75 μ M (10000 c.p.m./10 μ l) and sonicated for 30 min at 25 °C. PLC activity was performed in a final volume of 100 μ l. Assay incubation was initiated by adding 10–15 μ g of coated vesicles or brain membrane protein to a buffer containing 1 mM sodium deoxycholate, 100 mM NaCl, 40 mM LiCl, 1 mM EDTA and 50 mM Tris/HCl, pH 6.8, and 7.5 pmol [3H]PtdInsP2 (7500–10000 c.p.m.). All assays were performed in the presence of 300 nM of free calcium. Free Ca^{2+} concentrations were set using a Ca^{2+} -EGTA buffer system as described by Herrero et al. [27]. Samples were incubated for 10 min at 37 °C in the absence and in the presence of 100 μ M, guanosine 5'-[$\beta\gamma$ -imido]triphosphate (p[NH]ppG) and the corresponding ligands. The incubation was terminated by the addition of 360 μ l of chloroform/methanol/HCl (1:2:0.2 by volume) and putting the tubes on ice. Two phases were separated by addition of 120 μ l of 2 M KCl and 160 μ l of chloroform followed by vortexing and

centrifugation (8500 g for 3 min.). Aliquots of 250 μ l were removed from the upper phase to evaluate PtdInsP2 hydrolysis.

Pertussis toxin ADP-ribosylation

Pertussis toxin ADP-ribosylation was carried out as described earlier [9]. Coated vesicles were incubated with preactivated pertussis toxin (1 μ g pertussis toxin/100 μ g protein) or vehicle for 30 min at 30 °C in a final volume of 1 ml in 100 mM potassium phosphate buffer, pH 7.4, containing 1 mM EDTA, 2 mM $MgCl_2$, 2 mM NAD^+ , 1 mM ATP, 100 μ M GTP and protease inhibitors (50 μ g/ml leupeptin and 50 μ g/ml aprotinin). After incubation, samples were centrifuged at 100000 g for 30 min and coated vesicles were washed and resuspended in isolation buffer before PLC assays or used in a second toxin-catalysed ADP-ribosylation in the presence of [^{32}P]NAD $^+$, followed by electrophoresis as described previously [28].

Immunoblot analysis

Bovine brain coated vesicles and stripped vesicles (25–100 μ g of protein) were subjected to electrophoresis on 10% (7%, when anti-PLC was used) polyacrylamide gels in the presence of SDS and were electrophoretically transferred to nitrocellulose, blocked for 1 h with 5% (w/v) non-fat skimmed milk in PBS, pH 7.2, as described earlier [29]. The blots were probed with the following commercial antisera RM/1 (anti- G_{α}), AS/7 (anti- $G_{i1,2}$), GC/2 (anti- G_{α}) and the monoclonal anti-PLC β_1 . After washing with 0.3% Tween 20 in PBS, second goat anti-rabbit antiserum coupled to horseradish peroxidase was added (1:1000 in PBS) in the case of G-protein subunits. In the case of PLC antibody, a goat anti-mouse antiserum coupled to horseradish peroxidase was used. After washing, specific bands corresponding to G_{α} -protein subunits were detected using 4-chloro-1-naphthol as substrate. The band corresponding to PLC β_1 was visualized by use of a chemiluminescent reagent, using the ECL Kit from Amersham.

SDS/PAGE

Samples containing 25–50 μ g of protein were analysed on 10 or 7% PLC β_1 -polyacrylamide gels containing 0.1% SDS as described earlier [30].

Protein determination

Protein concentration was determined by the method of Lowry et al. [31], using BSA as standard.

RESULTS

Effect of excitatory amino acids on PLC activity in bovine brain coated vesicles

Coated vesicles have been purified by several steps of differential centrifugation combined with Sephacryl S-1000 chromatography as described previously [24]. This method yields a coated vesicle preparation with a typical electrophoretic profile (see Figure 2a) and minimal contamination [24]. Table 1 summarizes the data corresponding to PLC activity in the different fractions, measured in the absence (basal) and in the presence of 100 μ M p[NH]ppG and in the presence of p[NH]ppG plus 100 μ M of excitatory

Table 1 Effect of excitatory amino acids on [³H]PtdInsP₂ hydrolysis in bovine brain membranes and the different fractions obtained during coated vesicle purification from bovine brain

Membranes (10–15 µg) were incubated as described in the Materials and methods section in the presence of 300 nM free calcium and in the absence (basal) and presence of p[NH]ppG and p[NH]ppG plus the different compounds at 100 µM. Data, expressed as pmol/min per mg protein, are means ± S.E.M. of separate experiments (*n* is given in parentheses) performed in triplicate. * *P* < 0.01; ** *P* < 0.005; *** *P* < 0.0005 significantly different from the activity in the presence of 100 µM p[NH]ppG as determined with Student's *t* test.

		100 µM p[NH]ppG	100 µM p[NH]ppG + glutamate	100 µM p[NH]ppG + <i>trans</i> -ACPD	100 µM p[NH]ppG + carbachol
Bovine brain membranes	5.05 ± 0.75 (5)	6.44 ± 0.763 (5)	8.19 ± 0.58 (4)**	6.94 ± 1.13 (4)	7.97 ± 0.66 (4)**
20 000 g pellet	1.02 ± 0.002 (3)	3.4 ± 0.5 (3)	3.16 ± 0.66 (3)	4.69 ± 0.26 (3)*	4.7 ± 1.2 (3)
12.5% Ficoll–sucrose pellet	0.72 ± 0.053 (3)	3.22 ± 0.29 (3)	3.18 ± 0.25 (3)	5.00 ± 0.46 (3)**	4.37 ± 0.15 (3)**
100 000 g pellet	1.67 ± 0.13 (3)	2.47 ± 0.6 (3)	3.27 ± 0.06 (3)	3.82 ± 0.35 (3)*	3.72 ± 0.34 (3)*
Peak I	1.79 ± 0.58 (3)	7.02 ± 0.16 (3)	6.66 ± 0.85 (3)	6.48 ± 0.21 (3)*	5.45 ± 1.1 (3)
Peak II (coated vesicles)	1.88 ± 0.14 (7)	4.29 ± 0.28 (7)	3.05 ± 0.5 (5)**	2.35 ± 0.5 (7)***	2.79 ± 0.4 (3)***

Table 2 Effect of various neurotransmitter receptor agonists on p[NH]ppG-stimulated PLC activity in bovine brain coated vesicles

Bovine brain coated vesicles (10–15 µg) were incubated as described in the Materials and methods section in the presence of 300 nM free calcium and 100 µM p[NH]ppG and in the presence of 100 µM p[NH]ppG plus 100 µM of the indicated compounds. p[NH]ppG-stimulated PLC activity was 4.51 ± 0.25 pmol/min per mg protein. Values, expressed as a percentage with respect to p[NH]ppG-stimulated PLC activity, are means ± S.E.M. of three experiments performed in duplicate. * *P* < 0.05 and ** *P* < 0.01 significantly different from control activity as determined with Student's *t* test.

Agonist	p[NH]ppG-stimulated PLC activity (%)
+ L-GLU	61.1 ± 5.5**
+ <i>trans</i> -ACPD	56.6 ± 12.2**
+ QA	54.4 ± 13.2**
+ IB	72.2 ± 5.5**
+ CA	53.3 ± 8.8**
+ CA + <i>trans</i> -ACPD	38.8 ± 7.7**
+ QA + <i>trans</i> -ACPD	57.7 ± 13.3*
+ QA + CA	34.4 ± 5.5**

Table 3 Effects of mGluR antagonists on PLC inhibition by *trans*-ACPD

Bovine brain coated vesicles (10–15 µg) were incubated as described in the Materials and methods section and in the legend of Table 2 in the absence and presence of 100 µM *trans*-ACPD and in the absence and presence of 100 µM MCPG or AP3. Values are means ± S.E.M. of at least three independent experiments each performed in duplicate. * *P* < 0.0025 significantly different from control p[NH]ppG-stimulated PLC activity.

	PLC activity (pmol/mg protein per min)		
	Antagonist		
	None	+ MCPG	+ AP3
p[NH]ppG-stimulated	5.58 ± 0.22	4.70 ± 0.29	4.10 ± 0.72
+ <i>trans</i> -ACPD	2.91 ± 0.67*	4.42 ± 0.55	4.35 ± 0.13

amino acid (EAA) analogues (*trans*-ACPD and glutamate), or the muscarinic agonist carbachol, all of which have been described as PLC activators. As can be observed, p[NH]ppG stimulates PLC activity in all fractions. Glutamate and carbachol are able to stimulate PLC activity in the presence of p[NH]ppG in bovine brain membranes [32,33]. Under our conditions, *trans*-ACPD did not significantly alter PLC activity in membranes. When PLC activity was measured in the different fractions obtained at different stages of the coated vesicles purification-process, the effects of the neurotransmitter analogues used were significantly stimulatory except in the final coated vesicles fraction. As can be seen, p[NH]ppG-stimulated PLC activity in bovine brain coated vesicles was inhibited in the presence of 100 µM glutamate, *trans*-ACPD or carbachol. These data agree with our previous results where the presence of PLC activity in bovine brain coated vesicles was described as being regulated by guanine nucleotides in both an inhibitory and a stimulatory manner depending on the nucleotide concentration [9].

To assess further the presence of PLC activity in bovine brain coated vesicles and its putative coupling to mGluRs, the assays were performed using the different glutamate agonists that have been described as mGluR analogues [5]. As shown in Table 2, the

agonists studied produced inhibitory effects at 100 µM, with a ranked order of potency similar to that described by us previously where the characterization of mGluRs by radioligand binding experiments was reported [18,19]. All these inhibitory effects were dependent on the presence of p[NH]ppG and no variation with respect to basal PLC activity was observed in the absence of the guanine nucleotide (results not shown), suggesting the mediation of a G-protein. These data are consistent with a previous report [19] where it is demonstrated that mGluRs are regulated by GTP in coated vesicles. When we used carbachol as a muscarinic agonist, PLC activity was inhibited 45% in bovine brain coated vesicles. Inhibition of PLC activity by muscarinic analogues has been described in rat thyroid cells [14]. When this agonist was combined with QA or *trans*-ACPD, a more profound inhibition was obtained, 64 and 59% respectively, indicating that the muscarinic receptor analogue and the mGluR analogue act through different receptor pathways. In contrast with these results, when QA and *trans*-ACPD were combined no additive effects were observed. On the other hand, the inhibitory effects of *trans*-ACPD on p[NH]ppG-stimulated PLC were abolished by DL-AP3 and MCPG, two different specific antagonist of mGluR (Table 3).

It has been described that EAAs can inhibit stimulated turnover of phosphatidylinositol through ionotropic glutamate receptors in hippocampal slices [11]. In order to avoid the effect of this kind of receptor on the glutamate-induced inhibition of

Figure 1 Effect of pertussis toxin ADP-ribosylation on the inhibition of PLC activity by mGluR agonists

Bovine brain coated vesicles were incubated with preactivated pertussis toxin ($1 \mu\text{g}/100 \mu\text{g}$ protein) (●) or vehicle (○) for 30 min at 30°C before PLC activity assay, as described in the Materials and methods section. PLC activity was measured as described in the Materials and methods section and in the legend of Table 2 in the presence of p[NH]ppG and different concentrations of QA (a) and *trans*-ACPD (b). Data are means $\pm$ S.E.M. of three separate experiments performed in duplicate. Significant differences from PLC activity when coated vesicles were preincubated with pertussis toxin are: for QA, * $P < 0.01$ and ** $P < 0.001$; and for *trans*-ACPD, * $P < 0.005$ and ** $P < 0.01$. Inset shows pertussis toxin-catalysed ADP ribosylation of the remaining non-ADP ribosylated G-protein subunits after different preincubation times with the toxin.

PLC, we also performed the experiments in the presence of $100 \mu\text{M}$ MK-801 (an *N*-methyl-D-aspartate receptor antagonist) and $100 \mu\text{M}$ CNQX (a specific antagonist of kainate/AMPA receptors). The PLC activity observed under these conditions was similar to that obtained in the absence of the ionotropic glutamate receptor antagonists and *trans*-ACPD also inhibited p[NH]ppG-stimulated PLC activity by the same magnitude, confirming the above mentioned results (not shown).

Pertussis toxin effect on PLC activity in bovine brain coated vesicles

PLC activity in coated vesicles has been shown to be regulated by guanine nucleotides in a biphasic manner: inhibiting in the nanomolar range and stimulating in the micromolar range, suggesting that the enzyme is regulated by putative inhibitory and stimulatory G-proteins, respectively [9]. Given that the inhibitory pathway has been shown to be pertussis toxin-sensitive in bovine brain coated vesicles [9] and that pertussis toxin decreases the affinity of metabotropic glutamate binding sites for its agonist in coated vesicle preparations [19], the effects of this toxin on the inhibitory effects of glutamate agonist on p[NH]ppG-stimulated PLC activity were studied. In agreement with the data presented in Table 2, Figure 1 shows that both QA and *trans*-ACPD inhibited PLC activity in a dose-dependent manner in coated vesicles preincubated in the absence of pertussis toxin, QA being more potent than *trans*-ACPD, in accordance with the results shown above (Table 2). These inhibitory effects were abolished when coated vesicles were preincubated with pertussis toxin (Figure 1), suggesting the coupling of an mGluR, in an inhibitory manner, to a PLC activity through a pertussis toxin-sensitive G-protein. As shown in Table 4, pertussis toxin also blunted the inhibitory effects of carbachol in coated vesicles. Preincubation of coated vesicles with pertussis toxin under these conditions brings about ADP-ribosylation of a 40 kDa band.

Table 4 Pertussis toxin effect on carbachol inhibition of p[NH]ppG-stimulated PLC activity

Coated vesicles were incubated with preactivated pertussis toxin or vehicle for 30 min at 30°C . After this time PLC activity ($\text{pmol} \cdot \text{mg}^{-1} \cdot \text{min}^{-1}$) was measured in the presence of $100 \mu\text{M}$ p[NH]ppG and in the presence and absence of $100 \mu\text{M}$ carbachol. Values expressed as percentage with respect to p[NH]ppG-stimulated PLC activity, are means $\pm$ S.E.M. of three experiments performed in duplicate. * $P < 0.001$ significantly different from control preincubated with vehicle.

	PLC activity	
	(% of p[NH]ppG-stimulated activity)	
	– Pertussis toxin	+ Pertussis toxin
p[NH]ppG-stimulated PLC	4.6 ± 0.22 (100%)	4.42 ± 0.10 (100%)
+ Carbachol $100 \mu\text{M}$	2.88 ± 0.15 (60%)	4.21 ± 0.43 (95%)*

This was followed by a further pertussis toxin-catalysed ADP ribosylation using ^{32}P -labelled NAD^+ . As can be seen in the inset of Figure 1, the amount of radioactivity incorporated in the 40 kDa band decreases with incubation time, being barely detectable after 30 min of preincubation with the toxin. These results agree with those of Ros et al. [28] who described the presence of pertussis toxin substrates in coated vesicles.

Presence of G-proteins and PLC β_1 in bovine brain coated vesicles

Bacterial toxins from *Bordetella pertussis* and *Vibrio cholera* have been used previously to demonstrate the presence of G-proteins in bovine brain coated vesicles [28]. A band of 40 kDa, presumably $G_i\alpha$ and/or $G_o\alpha$ subunits, and two bands of 42 and

Figure 2 Electrophoretic profile of bovine brain coated vesicles and analysis of the presence of G-protein α -subunits by immunoblot

(a) Protein (50 µg) corresponding to coated vesicle fraction (PII) and stripped vesicles (PII-clt) were subjected to 10% PAGE in the presence of SDS and stained with Coomassie Blue. (b) Decoated vesicles (50 µg) were subjected to SDS/PAGE under the same conditions as described above, transferred electrophoretically to nitrocellulose and probed with AS/7 (anti G_iα_{1,2}), RM/1 (anti-G_sα) and GC/2 (anti-G_oα) antibodies as described in the Materials and methods section.

47 kDa, corresponding to G_sα forms, were described in coated vesicles as a result of pertussis and cholera toxin catalysed ADP-ribosylation respectively. In an approach to assess and identify the G-proteins present in coated vesicles, immunoblotting with specific antisera was carried out. Figure 2(a) shows the electrophoretic pattern of coated vesicles, PII corresponds to coated vesicles obtained from the Sephacryl S-1000 column, which contain the clathrin heavy chain (180 kDa) as the major component as well as the light chains (33–36 kDa) and the other two major family proteins (100–115 kDa; 50–55 kDa) implicated in clathrin assembly to the plasma membrane, termed adaptor proteins, and PII-Clt corresponds to the proteins of stripped vesicles, showing a relatively small quantity of the 180 kDa clathrin heavy chain. Since clathrin constitutes more than 50% of the coated vesicles protein content, we tried to eliminate most of it before the immunoblotting assay by using an alkaline buffer treatment [25]. Previous reports had shown that this gentle treatment maintains the functional integrity of coated vesicles [18]. Figure 2(b) shows immunoblots of decoated vesicles probed with AS/7, RM/1 and GC/2, polyclonal antibodies which recognizes G_iα_{1,2}, G_sα and αG_oα respectively. As can be observed, a band of 40 kDa is detected with AS/7 antibody. As this antiserum recognizes two proteins, G_iα₁ and G_iα₂, with very close molecular mass, the resulting band could represent one of them or a combination of both. The bands detected with RM/1 antibody confirmed the presence of G_sα forms in coated vesicles. These data agree well with those of Ozaki et al. [34] who also described the presence of G_i and G_s subunits in coated vesicles. On the other hand, antiserum GC/2 demonstrates the presence of G_oα (39 kDa), another pertussis toxin-sensitive G-protein and one of the most abundant G-proteins in the nervous central system.

Litosch et al. [17] have described G-protein-mediated inhibition of PLC in bovine brain solubilized membrane fraction, PLC β₁ being involved in this inhibition. Therefore, we have investigated the presence of this isoform in coated vesicles as a

Figure 3 Presence of PLC β₁ in bovine brain coated vesicles

Membranes (PI), coated vesicles (PII) and stripped coated vesicles (PII-clt) (25 µg of each) were subjected to 7% PAGE in the presence of SDS, transferred electrophoretically to nitrocellulose and probed with the isoform-specific monoclonal antibody against PLC β₁, as described in the Materials and methods section.

candidate to be involved in this inhibitory pathway. As can be observed in Figure 3, PLC β₁ was detected by immunoblot in bovine brain coated vesicles.

All these results suggest the presence in bovine brain coated vesicles of a PLC activity coupled to an mGluR in an inhibitory manner through a Pertussis toxin-sensitive G-protein and confirm the presence of several G-proteins and PLC β₁ in these intracellular organelles.

DISCUSSION

Evidence about the linkage of a single receptor subtype to multiple transduction pathways has recently been accumulated among the members of the G-protein-coupled receptors. At least seven mGluRs have been cloned, expressed in different cell lines and their coupling to transducing enzymes has been analysed [2–6]. So far, none of these receptors has been reported to inhibit PLC activity, whereas two products of the clones are positively coupled to the enzyme [2,5] and the others have been shown to be coupled to the inhibitory cascade of cyclic AMP generation [4,6].

In the present report we found a negative regulation of PLC activity in bovine brain coated vesicles. Under our experimental conditions, QA and *trans*-ACPD are able to inhibit the p[NH]ppG-stimulated hydrolysis of the phosphatidyl [2-³H]-inositol 4,5-bisphosphate ([³H]PtdInsP₂), in bovine brain coated vesicles (Tables 1 and 2). The inhibition of p[NH]ppG-stimulated PLC activity caused by *trans*-ACPD is still observed in the presence of MK-801 and CNQX, indicating that this effect is not mediated by ionotropic glutamate receptors, which have been proposed to act indirectly on the PLC activity [11]. On the other hand, DL-AP3 and MCPG, two different and selective antagonists of mGluR, abolished the inhibitory effects of the above mentioned glutamate metabotropic agonist (Table 3). The presence of mGluRs [19] and muscarinic receptors [35] has been described in bovine brain coated vesicles by pharmacological approaches. Although muscarinic receptors have been shown not to be coupled to G-proteins in coated vesicles [35], under our conditions carbachol inhibits p[NH]ppG-stimulated PLC activity

and this inhibition is additive to the inhibition produced by *trans*-ACPD and QA (Table 2). These data suggest that these neurotransmitters act through different receptor pathways and that this muscarinic receptor remains coupled in an inhibitory manner in coated vesicles. The existence of a muscarinic receptor coupled to PLC activity in an inhibitory manner has been previously described in rat thyroid cells [14]. The fact that pertussis toxin interferes with both pathways could be explained (besides the possible use of the same G-protein by both receptors) by the presence of several pertussis toxin substrates in coated vesicles, as demonstrated by immunoblot. Thus, each receptor might use different pertussis toxin-sensitive G-proteins.

Data using different mGluR agonists and dose–response curves (Table 2 and Figure 1) indicate that the pharmacological profile for the inhibition of PLC activity stimulated in the presence of p[NH]ppG correlates with the mGluR that we described by binding assays in bovine brain coated vesicles [18,19] and shows a similar pharmacology to that described by Tanabe et al. [4] for the mGluR₂ subtype coupled to the inhibitory cyclic AMP cascade. The range of concentrations needed to obtain inhibition by glutamate analogues in this preparation (Figure 2) is wider than in other reports where plasma membrane preparations are used, which could be for several reasons, such as the lack of specific analogues for the different mGluRs, the fact that the micro-environment of receptors and G-proteins play an important role in their coupling [36,37] and the possibility that receptor–G-protein coupling for different receptors might be differently affected upon their translocation from plasma membranes into coated vesicles. In this sense, pharmacological differences between adenosine A₁ receptors from plasma membrane and coated vesicles have been reported [37]. Otherwise different affinity states for mGluRs in bovine brain coated vesicles have been found [19]. This inhibitory receptor might be a candidate for a new subclass of mGluRs that could represent either a different protein (i.e. with a different sequence) or one of the already known receptors coupled to a different transducing enzyme in a manner not yet described in other systems and preparations. It is now recognized that large sets of receptors are coupled to effectors by more than one G-protein and that the same neurotransmitter may act through the same effector system in a stimulatory and inhibitory manner, depending on the cell type studied or the brain region [38].

As shown in Figure 3, PLC β_1 is present in coated vesicles. This enzyme has been shown to be inhibited through a G-protein-mediated mechanism in bovine brain solubilized membranes [17]. This makes PLC β_1 a good candidate as the effector system that is under negative control by *trans*-ACPD in coated vesicles. Nevertheless, further investigations would be necessary to elucidate this point.

A major finding of the present study is that PLC activity in coated vesicles is coupled to the mGluRs through a pertussis-toxin sensitive G-protein. The inhibition produced by QA and *trans*-ACPD was eliminated when bovine brain coated vesicles were treated with pertussis-toxin (Figure 1). These results and the dependence of PLC activity on GTP analogues [9] support the notion of the coupling of this system through a G-protein in coated vesicles and confirm previous results where an inhibitory pathway of the PLC in bovine brain coated vesicles was shown to be mediated by a pertussis toxin-sensitive G-protein [9]. Although the presence of pertussis toxin substrates of 40 kDa have been previously described [28], the present report shows by immunoblotting that G $\alpha_{1,2}$ and G α_o are present in bovine brain coated vesicles and hence are candidates to mediate the inhibitory action of glutamate and carbachol on stimulated PLC activity (Figure 2). On the other hand, as pertussis toxin does not affect

the stimulation of PLC in coated vesicles [19] and PLC β_1 has also been described as the effector for the G $_q$ /G $_{11}$ -mediated stimulation of PLC [39], it is possible that a member of this G-protein family also mediates stimulation in coated vesicles. Nevertheless, identification and/or direct correlation between receptor-mediated stimulation or inhibition of PLC and any known G-protein in coated vesicles remains to be elucidated.

The presence of the inhibitory signal transduction systems coupled to their receptors in bovine brain coated vesicles [24] could be explained on the basis of the tighter coupling of the inhibitory receptors to their G-proteins compared with the interaction of stimulatory receptors with their stimulatory G-proteins [40–42]. The presence of several G-protein coupled receptors in coated vesicles have been reported [22,24,43]. However, in contrast to the plasma membrane, only receptors that act by inhibiting the generation of second messengers have been reported to remain coupled to their effector system in these subcellular fractions [24]. This would agree with the lack of effect of *trans*-ACPD in plasma membranes, where its inhibitory action on PLC may be counterbalanced by the stimulatory effects of GTP analogues. In addition, it has been demonstrated that pertussis toxin-sensitive G $_i$ can be selectively depleted from the plasma membrane [44]. The fact that the inhibitory effects of glutamate analogues and carbachol are detected only in coated vesicles agrees with the above mentioned idea of a selective enrichment of the inhibitory components in intracellular compartments. This redistribution into intracellular compartments could be a general consequence of the internalization process in the regulation of many cell surface molecules. These selective effects and the observation that the inhibitory coupling remains in intracellular compartments, as well as the physiological meaning, remains an exciting field to be elucidated.

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