Presence of phospholipase C in coated vesicles from bovine brain

Dual regulatory effects of GTP-analogs

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Bovine brain coated vesicles display free calcium-dependent phospholipase C activity. Gpp(NH)p and GTPγS inhibited phospholipase C at nanomolar concentrations. Increasing concentrations of Gpp(NH)p and GTPγS reversed the inhibitory effects and stimulated phospholipase C activity. Preincubation of coated vesicles with pertussis toxin blocked the poorly-hydrolyzable GTP-analogs' inhibitory effects on phospholipase C. These data indicate that guanine nucleotides exert a dual regulatory control of phospholipase C in coated vesicles and that the inhibitory pathway is mediated by a pertussis toxin-sensitive G-protein.

Coated vesicle; Phospholipase C; G-protein; Guanine nucleotide

1. INTRODUCTION

Phospholipase C (PLC) is a virtually ubiquitous membrane-associated transducing enzyme. Its activation through hormones and/or neurotransmitters leads to the breakdown of phosphatidylinositol phosphates, generating second messengers such as inositol phosphates and diacylglycerol [1]. Inositol phosphates trigger calcium release from intracellular compartments [2], and diacylglycerol stimulates calcium-dependent protein kinase C [3]. Activation of PLC by hormones and neurotransmitters has been shown to be mediated by guanine nucleotide binding proteins [4,5]. In the same cells, this process seems to be pertussis toxin-sensitive [6,7] while in others it is not [8,9]. The G-proteins G_z [10] and members of the G_q family [11,12], which are not substrates for pertussis toxin, have been proposed to be candidates for the G-protein that mediates pertussis toxin-insensitive stimulation of PLC. On the other hand, several studies with membranes [13] and permeabilized cells [14,15] have suggested that PLC is also under the control of a G-protein-mediated inhibitory pathway. In analogy with the adenylate cyclase, which is regulated by two different G-proteins, G_s (stimulatory) and G_i (inhibitory), and in spite of the lack of a precise characterization of the G-proteins responsible for stimulation and inhibition of PLC, the terms Gp_s and Gp_i have been assigned.

Recently, evidence for the presence of the components of the adenylate cyclase system such as receptors that mediate stimulation [16], receptors that mediate inhibition [17,18], the catalytic activity and G-proteins [18,19] have been described in coated vesicles. These are subcellular organelles involved in endocytotic and exocytotic protein trafficking within the cell [20]. The presence of transducing systems in compartments besides plasma membrane opens several questions about signal transduction, desensitization and protein trafficking. In the present report, the presence of PLC and its dual regulatory control by putative stimulatory and inhibitory G-proteins in coated vesicles are investigated.

2. MATERIAL AND METHODS

2.1. *Materials*

Phosphatidyl [2-³H]inositol 4,5-bisphosphate ([³H]Ptd Ins P_2) was purchased from Amersham. Pertussis toxin was from List Biological Laboratories. Non-hydrolysable guanine nucleotides were purchased from Boehringer-Mannheim. All other reagents were of analytical grade.

2.2. *Preparation of membranes and coated vesicles*

Plasma membranes were obtained from bovine brain as described by Olsen et al. [21] and Recasens et al. [22]. Coated vesicles were obtained by Shephacryl S 1000 gel filtration as described by Bar-Zvi and Branton [23] and modified by González-Calero et al. [18]. This method yields a coated-vesicle preparation with minimal cross-contamination with other subcellular fractions as deduced by marker enzyme activities and electron microscopy [18].
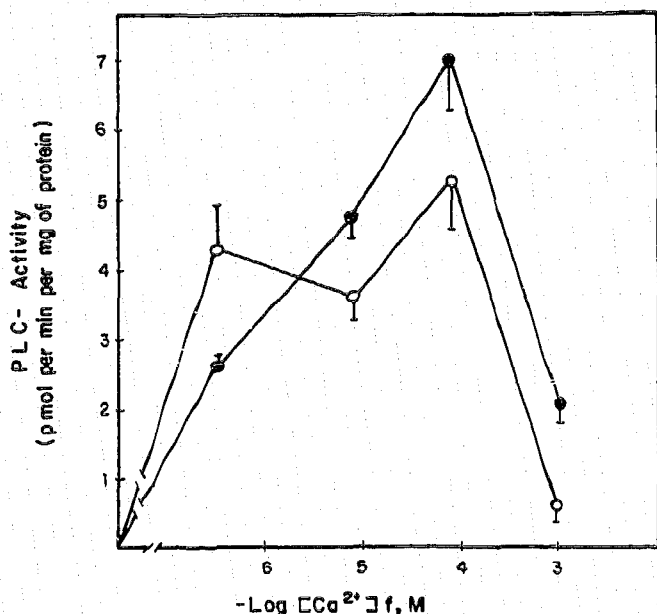
Fig. 1. Effects of free calcium concentration on phospholipase C activity. Coated vesicles (10–15 μg protein) were incubated with [³H]Ptd Ins P₂ for 10 min at 37°C as described in section 2 with (○) or without (●) 100 μM GTPγS. Data values are means ± SEM of three separate experiments performed in duplicate.

2.3. Phospholipase C assay

Phospholipase C in coated vesicles and plasma membranes was assayed in the presence of exogenous [³H]Ptd Ins P₂ as described by Tiger et al. [24]. Ptd Ins P₂ was dried under an N₂ stream, dissolved in 40 mM sodium deoxicholate, 50 mM Tris-HCl pH 6.8, sonicated and used immediately for assay as described earlier [24]. Phospholipase C assay was carried out for 10 min at 37°C, incubating 15 pmol [³H]Ptd Ins P₂ (15 000–20 000 cpm) with or without 10–15 μg of coated vesicles or plasma membrane protein in 100 μl of buffer containing 40 mM sodium deoxicholate, 100 mM NaCl, 40 mM LiCl, 1 mM EDTA and 50 mM Tris-HCl pH 6.8 and the indicated concentrations of free calcium. Free Ca²⁺ concentrations were set using a Ca²⁻-EGTA buffer system as described by Herrero et al. [25]. The incubation was terminated by the addition of 360 μm of chloroform/methanol/HCl (1:2:0.2, v/v) and putting the tubes on ice. After addition of 120 μl 2 M KCl and 160 μl of chloroform. The upper phase was used to evaluate Ptd Ins P₂ hydrolysis as described earlier [24].

2.4. Pertusis toxin ADP-ribosylation

Coated vesicles were incubated with preactivated pertussis toxin (1 μg/100 μg protein) or vehicle for 30 min at 30°C as described by Malbon et al. [26] in a final volume of 1 ml 100 mM phosphate buffer pH 7.5 containing 1 mM EDTA, 2 mM MgCl₂, 2 mM NAD⁺, 1 mM ATP, 100 μM GTP and protease inhibitors (leupeptin 50 μg/ml, aprotinin 50 μg/ml). After incubation, samples were centrifugated at 100 000 × g for 30 min and coated vesicles were washed and resuspended in isolation buffer, prior to PLC assay.

2.5. Protein determination

Protein concentration was determined by the method of Lowry et al. [27], using bovine serum albumin as a standard.

3. RESULTS AND DISCUSSION

Coated vesicles, isolated from bovine brain, display PLC activity. Fig. 1 shows that this activity is dependent

on the free calcium concentration in a similar manner to that described in plasma membranes from several tissues [25,28]. Maximal activity was observed at 80 μM free calcium, both in the presence and in the absence of GTPγS. Reduction of the free calcium concentration below 0.3 μM or an increase up to 1 mM led to a loss of activity. Taken into the account that the PLC activity found in the coated vesicles fraction is similar to that found in the plasma membrane fraction (Table I) and that cross-contamination in the coated-vesicle fraction is minimal [18], these data clearly indicate the presence of PLC in coated vesicles.

As PLC was shown to be stimulated through G-proteins in plasma membranes from different cell types [4,5], the effects of guanine nucleotides on PLC have been investigated in coated vesicles. Table II shows that poorly hydrolysable GTP analogs stimulated PLC, suggesting that a G-protein also mediates the activation of this enzyme in coated vesicles. This effect seems to be specific, since ATP did not stimulate PLC. As in the case of adenylate cyclase, a G-protein-mediated inhibitory pathway also has been described to regulate PLC activity [13–15]. This pathway was shown to be sensitive to lower concentrations of GTP-analogs [13] and in-

Table I

Phospholipase C activity in plasma membranes and coated vesicles.

Fraction	PLC activity (pmol/mg prot × min)	
	Basal	Gpp(NH)p-stimulated
Plasma membranes	3.68 ± 0.22	6.95 ± 0.33
Coated vesicles	2.87 ± 0.19	5.17 ± 0.21

10–15 μg of plasma membranes or coated vesicles protein were incubated with 15 pmol of [³H]Ptd Ins P₂ as described in section 2. Data values are means ± SEM of five separate experiments performed in triplicate.

Table II

Effects of nucleotides on phospholipase C activity in coated vesicles.

Nucleotide	PLC activity (% basal)
Control	100%
Gpp(NH)p	169 ± 19%
GTPγS	193 ± 46%
ATP	99 ± 1%

Phospholipase C activity was measured in the absence and in the presence of 100 μM of the indicated nucleotides as described in section 2. Control activity was 3.28 ± 0.39 pmol/mg prot × min. Data values are means ± SEM of four independent experiments performed in duplicate.
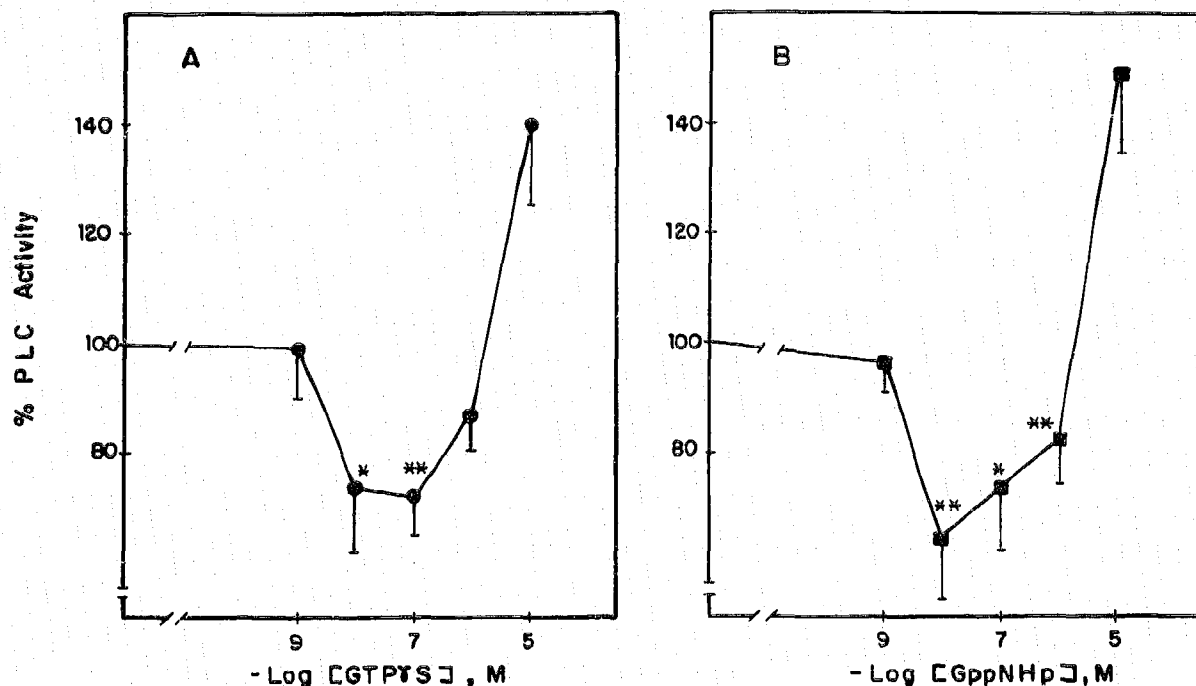
Fig. 2. Effects of poorly hydrolysable GTP analogs on phospholipase C activity. Coated vesicles (10–15 μg of protein) were incubated in the presence of 300 nM free calcium and the presence of the indicated concentrations of GTPγS (A) and Gpp(NH)p (B) as described in section 2. Basal phospholipase activity was 3.28 ± 0.39 pmol/mg prot × min. Data values are means ± SEM of three separate experiments performed in duplicate. $*P < 0.05$; $**P < 0.02$; significantly different from basal activity.

dependent of calcium mobilization or cAMP generation [14,15]. In Fig. 2 it can be observed that nmolar concentrations of Gpp(NH)p and GTPγS significantly inhibit PLC activity, while μmolar concentrations reverse the inhibitory effects of poorly hydrolysable GTP analogs. A stimulation of 50% and 40% was observed at 10 μM Gpp(NH)p and 10 μM GTPγS, respectively. These data show that PLC in coated vesicles behaves in a similar fashion to that described in membranes [13] and clearly suggest a dual, G-protein-mediated, regulatory control of PLC in this subcellular fraction.

Stimulation [4,5] and inhibition [13–15] of PLC have been described to be pertussis toxin-sensitive processes, depending on the cell type studied. In order to investigate whether guanine nucleotide stimulation and/or inhibition of PLC are sensitive to pertussis toxin, the effects of GTP analogs were tested in coated vesicles preincubated with or without preactivated pertussis toxin. Fig. 3 shows that the inhibitory effects of Gpp(NH)p and GTPγS on PLC at nmolar concentrations were suppressed by pertussis toxin, suggesting that the putative G-protein that mediates inhibition in coated vesicles is a substrate for pertussis toxin. The fact that basal PLC activity in coated vesicles incubated with pertussis toxin is higher than in controls (Fig. 3, legend) also supports the idea that the inhibitory pathway is mediated by a pertussis toxin-sensitive G-protein. In agreement with these data, the presence of pertussis toxin substrates has been demonstrated in coated

vesicles [19]. On the other hand, Fig. 3 also shows that pertussis toxin does not affect the stimulation of PLC caused by GTP analogs. These data support the presence of a pertussis toxin-insensitive G-protein coupled to PLC in a stimulatory manner. Due to the existence of a growing family of G-proteins, substrates [29,30] (or not [31]) for pertussis toxin-catalized ADP-ribosylation, as well as the existence of several forms of PLC [32], identification of which G-protein couples to which PLC isoform has not yet been resolved. Nevertheless, recently, evidence suggesting that Gq is the G-protein which mediates pertussis toxin-insensitive stimulation of PLC activity has been shown [33,34].

In the present report, besides the presence of PLC activity in coated vesicles isolated from bovine brain, a dual regulatory effect of GTP-analogs on this signal-transducing enzyme has been described in this subcellular fraction. Thus, as described in membranes [13] and in an analogous fashion to the adenylate cyclase, in coated vesicles, PLC seems to be under the control of both stimulatory and inhibitory pathways mediated by stimulatory and inhibitory G-proteins, respectively.

The physiological meaning of the presence of membrane-associated transducing enzymes such as adenylate cyclase or PLC in coated vesicles remains to be elucidated. The presence of transducing enzymes in coated vesicles might be understood in the context of desensitization. Thus, as receptors, G-proteins which have been shown to be present in subcellular fractions
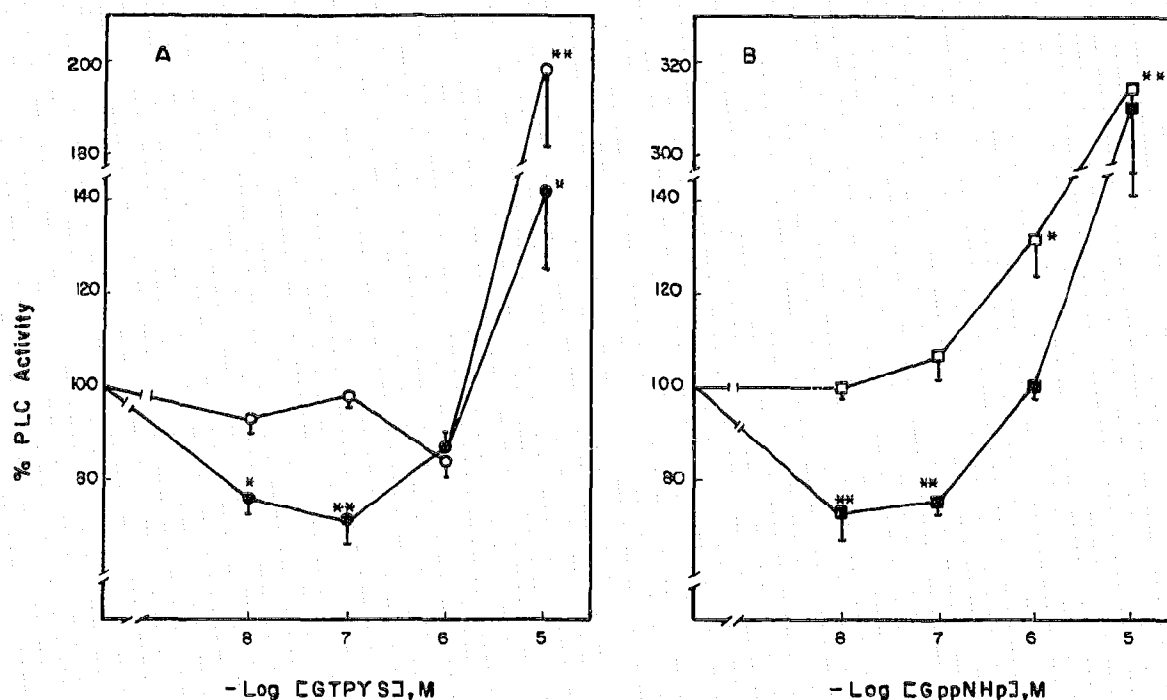
Fig. 3. Pertussis toxin effects on poorly hydrolysable GTP analogs modulation of phospholipase C activity. Coated vesicles were incubated with preactivated pertussis toxin (1 μg/100 μg protein (O, □) or vehicle (●, ■) for 30 min at 30°C prior to PLC activity assay as described in section 2. Basal PLC activities were 2.4 ± 0.67 and 4.23 ± 0.98 pmol/mg prot × min, in the absence and in the presence of pertussis toxin, respectively. Data values are means + SEM of three separate experiments performed in duplicate. *P < 0.05; **P < 0.02; significantly different from its basal activity.

after agonist–receptor interactions [35], transducing enzymes might also be internalized. Another possibility would be the continuation of its transducing function next to the plasma membrane. This possibility has been suggested earlier [35] and in this sense, internalization of middle t has been shown to be necessary to produce transformation [36]. Further studies to identify the G-proteins present in coated vesicles and their putative role in signal transduction, protein trafficking and/or desensitization will be needed.

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