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Activation of phosphatidylinositol lipid-specific phospholipase C-β_3 by G-protein $\beta\gamma$ subunits

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A novel member of the inositol lipid-specific phospholipase C family, PtdIns-PLCβ_3, is shown to be activated by $\beta\gamma$ subunits of the heterotrimeric GTP-binding protein, transducin. The activation is a direct effect since it is observed with the purified proteins. Furthermore, the activation is blocked by the GDP-liganded α subunit of transducin, confirming that the effect is due to free $\beta\gamma$ subunits. The implications with respect to receptor–PtdIns-PLC coupling are discussed.

Phospholipase C activation; G-protein $\beta\gamma$ subunit; Transducin

1. INTRODUCTION

Hormone and growth factor stimulation of inositol lipid hydrolysis has been shown to occur via at least two distinct general mechanisms that relate to particular members of the phosphoinositide-specific phospholipase C (PtdIns-PLC) family. Thus, it is established that the PtdIns-PLCγ family is activated by receptor complex formation and tyrosine phosphorylation (reviewed in [1,2]), while the PtdIns-PLCβ family is regulated by interaction with Gα.GTP subunits of the pertussis toxin insensitive Gq class of heterotrimeric G-protein [3,4]. This latter mode of activation itself shows specificity in that while $G\alpha_{q,11,14,16}$ will activate PtdIns-PLCβ_1, the related PtdIns-PLCβ_2 is more selectively activated by $G\alpha_{16}$ [5].

A further mode of activation has been sought for PtdIns-PLC enzymes in view of the fact that a number of hormones stimulate inositol lipid hydrolysis in a pertussis toxin sensitive manner (reviewed in [6]). Recent evidence suggests that the pertussis toxin sensitive coupling might be effected through G-protein $\beta\gamma$ subunit activation of PtdIns-PLC [7]. This mode of activation also shows specificity with respect to PtdIns-PLC proteins, with β_2 being much more effectively activated by $\beta\gamma$ subunits of retinal transducin than β_1 [8]. We have recently described the identification and isolation of a third β-family member (PtdIns-PLCβ_3) [9] and here the activation of this protein by $\beta\gamma$ subunits from transducin ($\beta\gamma_t$) is investigated.

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2. MATERIALS AND METHODS

2.1. *Purification procedures*

PtdIns-PLCβ_3 was purified to homogeneity as documented previously [9]. The $\beta\gamma$ subunits from transducin were purified as outlined in [7]. PtdIns-PLCβ_1 was purified (~50% pure) from bovine brain cytosol in a procedure similar initially to that outlined previously [10] in that the cytosol was subjected to acid precipitation with acetic acid, and then the resolubilized protein was subjected at pH 7.4 to anion-exchange chromatography using DE-52 (Whatman, UK). The method then employed heparin Sepharose chromatography followed by Mono Q and then Mono S column chromatography at pH 7.5 and pH 5.5, respectively, as described previously [9]. Phospholipase C activity was monitored throughout the purification using monolipid vesicles of $PtdInsP_2$ in the presence of 0.6% sodium cholate as detailed in [9].

2.2. *Western analysis*

Western analysis of the phospholipase C was performed as outlined previously [9] using monospecific polyclonal antisera (which in the case of the PtdIns-PLCβ_3 antisera has been affinity purified) and the ECL detection system (Amersham International, UK).

2.3. *The assay for activation of PtdIns-PLC by $\beta\gamma_t$*

This assay was largely as outlined [7]. Briefly, the lipids $PtdIns4,5P_2$ (Sigma Chemical Co., UK) and phosphatidylethanolamine (Lipid Products, UK) were mixed in a molar ratio of 1:10, together with [2-^{3}H]$PtdInsP_2$ (Amersham International, UK) to give a specific activity of 9.1 nCi/nmol $PtdIns4,5P_2$ and dried under N_2 before being dispersed by sonication into an aqueous buffer such that the final $PtdInsP_2$ concentration in the assay was 25 μM. The buffer composition was such that final concentrations in the assay were 50 mM Tris-maleate pH 7.5, 50 mM KCl, 10 mM 2,3-bisphosphoglycerate, 10 mM LiCl, 0.04% (w/v) sodium deoxycholate and 10 mM EDTA. Each assay tube received 10 μl of 62.9 mM $CaCl_2$, calculated to give a final free calcium concentration of 100 nM, 15 μl $\beta\gamma_t$ subunits at the concentrations indicated, 5 μl of phospholipase C, diluted in 10 mM Tris-HCl pH 7.5, 1 mM EDTA (so that each tube received 0.5 units of activity as judged by the monolipid, cholate assay: 1 unit = 1 nmol $PtdIns4,5P_2$ hydrolysed/min) and the reaction was begun with 40 μl of the lipid mixture. The reaction proceeded at 25°C for 10 min before termination by the addition of 260 μl of $CHCl_3$/MeOH/conc. HCl (100:100:0.6, v/v/v) and 75 μl of 1 M HCl. The tubes were then vortex-

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mixed and subjected to centrifugation to facilitate phase separation prior to removal of 175 μl of the aqueous phase for scintillation counting.

2.4. *Inhibition of $\beta\gamma_t$ activation by the $G\alpha_t \cdot$ GDP subunit*

These assays were performed as for the $\beta\gamma_t$ activation studies with the exception that the concentration of $\beta\gamma_t$ used was set at a final value of 1.5 μM, delivered in a 10 μl volume and that various concentrations of the $G\alpha_t \cdot$ GDP (in a 5 μl volume) were preincubated with the $\beta\gamma$ subunits and phospholipase at room temperature for 5 min before commencement of the reaction.

3. RESULTS

PtdIns-PLCβ_3 was purified as described previously to generate a single band on a Coomassie-stained gel [9]. However, in view of the established activation of the related PtdIns-PLCβ_2 by $\beta\gamma_t$ subunits [8], it was essential to confirm the antigenic purity of the β_3 preparation. Fig. 1 shows a Western blot with β_1-, β_2- and β_3-specific sera confirming that the PtdIns-PLCβ_3 preparation is devoid of both β_1 and β_2.

Activation of the PtdIns-PLCβ_3 by $\beta\gamma_t$ subunits was determined under conditions previously shown to be effective for PtdIns-PLCβ_2 [8]. It can be seen from Fig. 2 that the purified PtdIns-PLCβ_3 is indeed sensitive to activation by $\beta\gamma_t$ subunits and that marginal activation is seen for purified PtdIns-PLCβ_1 despite the fact that equivalent phospholipase activity, as judged by the cholate monolipid assay, was used for each isozyme (in some instances $\beta\gamma_t$ activation of PLCβ_1 was barely detectable). The dose–response for activation is similar to that seen for PtdIns-PLCβ_2 [8], although the latter studies were carried out in crude extracts.

In order to confirm that activation is due to the direct action of $\beta\gamma_t$ subunits on the PtdIns-PLC and not due to sequestration of some contaminating inhibitory Gα subunits, the action of $G\alpha_t \cdot$ GDP was investigated. It is evident from Fig. 3 that increasing the $G\alpha_t \cdot$ GDP concentration titrates out the activation of PtdIns-PLCβ_3 by $\beta\gamma_t$ subunits, consistent with the binding and sequestration of $\beta\gamma_t$.

4. DISCUSSION

It is demonstrated here that PtdIns-PLCβ_3 can be activated by free $\beta\gamma_t$ subunits. Importantly, this activation is achieved with purified components and is shown to be prevented by $G\alpha_t \cdot$ GDP. Previous studies on $\beta\gamma$ activation of PtdIns-PLC have been carried out on partially purified enzymes of unknown antigenicity [7] or crude extracts from cells transfected with specific PtdIns-PLC cDNAs [8]. It has thus not been possible, to date, to vigorously demonstrate that $\beta\gamma$ activation is a direct consequence of PtdIns-PLC interaction as opposed to effects on associated PtdIns-PLC components. The data here indicate that $\beta\gamma_t$ subunits interact directly with PtdIns-PLCβ_2.

The specificity of $\beta\gamma_t$ activation is evidenced here by the very weak effect on the PtdIns-PLCβ_1 enzyme.

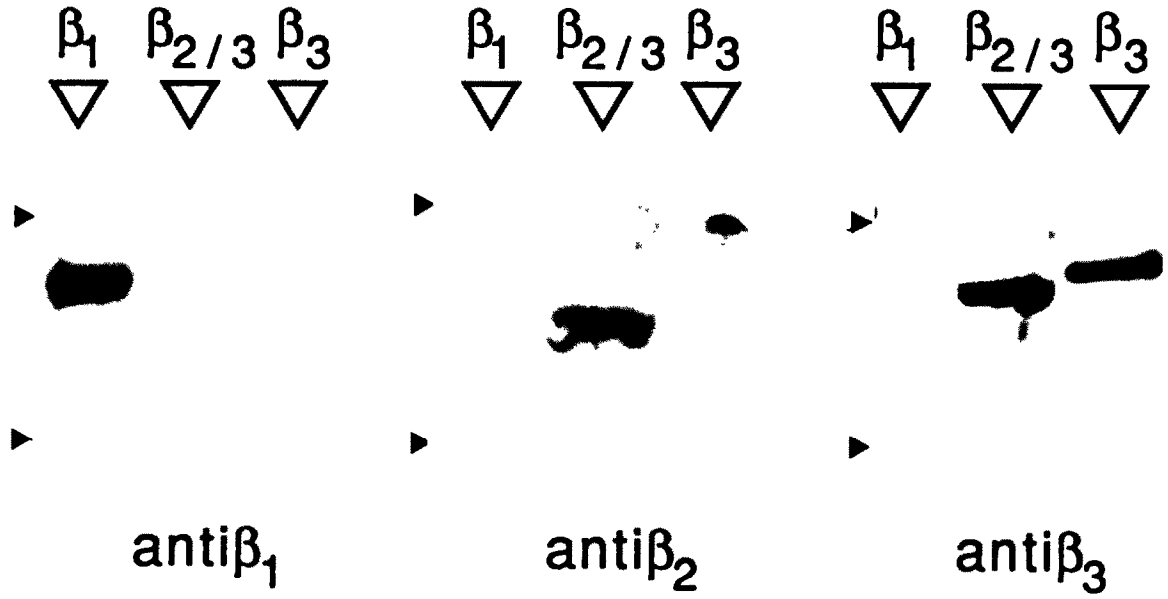

Fig. 1. Western analysis of phospholipases C of the β class. Samples subjected to polyacrylamide gel electrophoresis were loaded as indicated above each lane; β_1 = PtdInsPLCβ_1 purified from bovine brain; β_2/β_3 = a cellular extract of COS-1 cells transfected with a plasmid containing the PtdInsPLCβ_2 gene. Previous data (unpublished) has shown that untransfected COS-1 cells contain significant levels of a protein immunoreactive with antisera directed against PtdInsPLCβ_3 which is assumed to be endogenous PtdInsPLCβ_3; β_3 = PtdInsPLCβ_3 purified from HeLa cells. Samples transferred to immobilon-P were probed with polyclonal antipeptide antisera specific for individual PtdInsPLCβ family members as indicated below each panel. Co-electrophoresed molecular weight standards, represented by triangles on the left of each panel represent 200 kDa and 116.25 kDa in descending order.

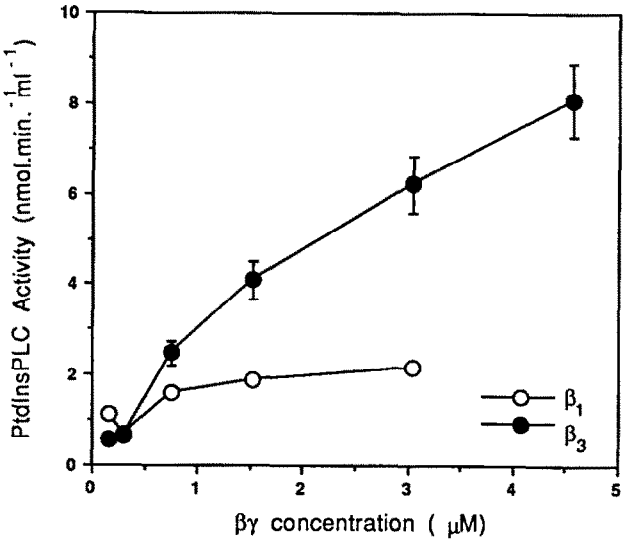

Fig. 2. Activation of PtdInsPLCβ_1 and β_3 by $\beta\gamma_t$ subunits. Identical amounts of PtdInsPLCβ_1 and β_3 activity (as assessed by a standard cholate assay) were incubated with varying amounts of $\beta\gamma_t$ subunits, as indicated, and the phospholipase activity was monitored under the conditions outlined in section 2. This is one of two complete titration experiments, both of which gave similar results.

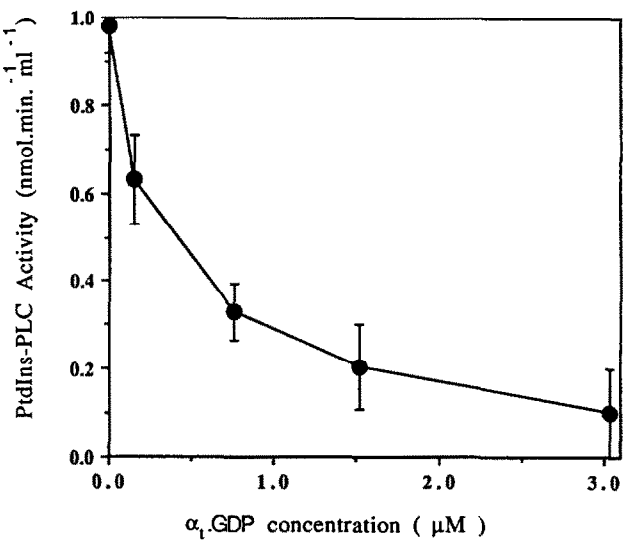

Fig. 3. Inhibition by $G\alpha_t \cdot GDP$ of the activation of PtdInsPLCβ_3 by $\beta\gamma_t$ subunits. Fixed amounts of PtdInsPLCβ_3 and $\beta\gamma_t$ subunits (to give a final $\beta\gamma_t$ concentration of approx. 1.5 μM) were incubated with varying amounts of $G\alpha_t \cdot GDP$ and the phospholipase activity assessed. Results represent a typical experiment with the error bars denoting the range of duplicates.

These and previous results show that, of the known members of the PtdIns-PLCβ class, both β_2 and β_3, show sensitivity to $\beta\gamma_t$ while β_1 is only poorly responsive. Although, under the conditions used, the activation of PtdIns-PLCβ_3 (and β_2) is only achieved at rather high concentrations of $\beta\gamma_t$, the activation is very sensitive to lipid context (unpublished). In addition, it is conceivable that different $\beta\gamma$ subunit combinations may be capable of activating PtdInsPLCβ family members to differing extents. Thus, the sensitivity of these enzymes to $\beta\gamma$ in an in vivo context may well differ from that described here.

As discussed previously [7,8], the activation of PtdIns-PLC enzymes by $\beta\gamma$ subunits provides a rationale for the pertussis toxin sensitive pathway of agonist induced inositol lipid hydrolysis. The studies described here identify PtdIns-PLCβ_3 as a target for $\beta\gamma_t$ activation and therefore suggest that pertussis toxin sensitive signalling may be channelled not only through the β_2 enzyme [8], but also through PtdIns-PLCβ_3.

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